Glycosylation Properties Associated with Development and Differentiation of Spermatogonial Stem Cells in Mammalian Testis(Dissertation_全文)

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ABSTRACT

The studies of this thesis investigated the distribution of a glycan epitopes *alpha-N-acetylgalactosamine* (GalNAc), which is recognized by lectin *Dolichos biflorus agglutinin* (DBA), on developing gonocytes at the early postnatal stage for definition of the initial differentiation process in the mouse testis. The present study also examined the role of GalNAc residues on cultured gonocytes associated with cell adhesion, proliferation, a pluripotent potential in domestic animal species.

The affinity of DBA binding to GalNAc residues were effectively improved by the heat-mediated antigen retrieval (HMAR) technique on germ cell population. In particular, the presence of GalNAc residues on gonocytes were firstly detected using the lectin DBA in the mouse testis. These results suggested that the affinity of DBA binding to gonocytes is a common molecular feature in mammalian species. We also found that the distribution of

these glycan epitopes depended on the type of developing gem cells. These molecular characteristics of glycan epitopes suggest that the identification and characterization of gonocytes using DBA may provide useful information to explorer the differentiation process of gonocytes associated with the stage-specific glycosylation of the terminal carbohydrate chain in mouse testis.

This study examined the distribution of GalNAc residues on the initial transition processes of gonocytes, suggesting that DBA specifically recognized glycan epitopes on gonocytes at 0.5 to 1 day postpartum (dpp), but was gradually disappeared at around 3-5 dpp. The loss of DBA affinity to gonocytes may arise from the change of glycosylation pattern, which associated with a physiological alteration of cell characteristics, may reflect the initiation of gonocyte differentiation. At this stage, some gonocytes were shown that DBA signals were intensely observed as condensed dots in the cytoplasm. Interestingly, these populations were progressively lost the expression of UCHL1 at around 5 to 7 dpp, and then they expressed a premeiotic marker STRA8 at 7 dpp, suggesting that the change of glycosylation on gonocytes coincide with the appearance of differentiating spermatogonia, which are progressed further differentiating stage for the onset of meiosis. Meanwhile, DBA-negative cells expressed UCHL1, but did not express STRA8 at the same stage. These molecular features indicate that gonocytes consist of a heterogeneous population, which seem to follow the different cell fate during the initial differentiation process of gonocytes.

This study examined how cell surface molecules such as GalNAc residues of glycoproteins affect the adhesion and proliferation of gonocytes in cultured bovine gonocytes. The result showed that gonocytes from the bovine testis formed mouse embryonic stem (ES)-like cell colonies on plates, which were previously coated with DBA or extracellular matrix

(ECM) components (gelatin (GN), laminin (LN)) or an artificial ECM component, poly-L-Lysine (PLL). However, the number of colonies on the DBA plate was significantly higher than the numbers of colonies on the GN, LN and PLL plates. The DBA plate effectively suppressed the growing of somatic cells, which supported to increase colony formation. Pretreating gonocytes with DBA to neutralize the terminal GalNAc residues strongly suppressed colony formation. The expressions of a germ cell-specific gene and pluripotency-related transcription factors were increased considerably on the DBA plates, indicating that the DBA-coated plates support proliferation of gonocytes during culture. These results suggest that the GalNAc residues on the surface of gonocytes can recognize pre-coated DBA on culture dishes. DBA supports cell adhesion and induces the proliferation of gonocytes by the formation of GalNAc-DBA complexes, which are associated with the increase of pluripotent gene expressions *in vitro*. These glycan complexes through GalNAc epitopes may provide a suitable microenvironment for the adhesion and colony formation of primitive germ cells in culture.

The results described above suggest that the precise definition of GalNAc residues on the development of gonocytes may provide useful information to understand the initial differentiation process of spermatogenesis in the mammalian species. These glycan epitopes on the surface of germ cells may play a role in the cell interaction and cell survival in the culture system.

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LIST OF ABBREVIATIONS

A_{al} spermatogonia	Alined type A spermatogonia
ANOVA	Analysis of variance
A_{pair} spermatogonia	Paired type A spermatogonia
A_s spermatogonia	Single type A spermatogonia
bFGF	Basic fibroblast growth factor
DAB	3, 3'- diaminobenzidine tetrahydrochloride
DBA	*Dolichos biflorus agglutinin*
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EG cells	Embryonic germ cells
EGF	Epidermal growth factor
ES cells	Embryonic stem cells
FACS	Fluorescence activated cell sorting
GalNAc	*N-acetylgalactosamine*
GDNF	Glial cell line-derived neurotrophic factor
GFRα1	GDNF family receptor α1
GluNAc	*N-acetylglucosamine*
GN	Gelatin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMAR	Heat-mediated antigen retrieval

LN	Laminin
MACS	Magnetic-activated cell sorting
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PGCs	Primordial germ cells
PLL	Poly-L-lysine
PLZF1	Promyelocytic leukemia zinc finger protein 1
POU5F1	POU domain, class 5, transcription factor 1
RT-PCR	Reverse transcription polymerase chain reaction
SSCs	Spermatogonial stem cells
TBS	Tris buffered saline
UCHL1	Ubiquitin carboxy-terminal hydrolase L1

CHAPTER 1

1. General Introduction

Spermatogenesis that is a unique productive process of male germ cells that continuously produce millions of spermatozoa every day in mammalian testis (Meistrich and van Beek, 1993). The foundation of this system is spermatogonial stem cells (SSCs) that have the ability to transmit genetic information to the next generation and the stem cell potential for the maintenance of a SSC pool throughout the reproductive life of mammals. These cells are generated from subpopulations of gonocytes in neonatal developing testis and contribute the initiation of spermatogenesis. Therefore, gonocyte that are present in the neonatal testis are primitive germ cells committed to male germline development. To understand the reproductive system of male germ cells, the study on the characteristics and the functional role of gonocytes during the neonatal period is critically important in the mammalian species.

Gonocytes in the neonatal testis are located on the central part of the seminiferous tubules (Clermont and Perey, 1957a) and are mitotically quiescent state, which exhibits to be arrested in the G0/G1 phase of the cell cycle (Hilscher and Makoski, 1968). During the early postnatal period, gonocytes resume mitotic division and were relocated toward the basement membrane of the seminiferous tubule (Clermont and Perey, 1957a; Huckins and Clermont, 1968; McLean et al., 2003). After translocation, gonocytes are eventually committed to spermatogenesis in the neonatal developing testis. Primitive spermatogonial populations in the postnatal testis are referred as the undifferentiated spermatogonia, which correspond to A_{single}, A_{pair} and $A_{aligned}$ spermatogonia (de Rooij, 2001; de Rooij and Russell, 2000; Nishimune et al., 1978), are differentiated differentiating spermatogonia (A_1 to A_4, Intermediated (In) and B spermatogonia). Subsequently, they differentiated into meiotic

spermatocytes, spermatid and spermatozoa. However, the initial differentiation processes of gonocytes and the appearance of SSCs remain unclear because of the difficulties in precise definition of germ cell populations in the early stage of developing testis. Therefore, there is a need for a specific marker that can distinguish multiple steps in the early germ cell development during the early neonatal period.

Stage-specific markers have been used the characterization, identification and purification of germ cells from distinct cell populations. It can be useful to explorer the regulatory mechanism in the differentiation of germ cells during spermatogenesis. During spermatogenesis, the transition of undifferentiated spermatogonia to differentiating spermatogonia involves in a cell surface molecule, which has known as c-Kit, a receptor for tyrosine kinase, indicating that a cell surface marker can contribute to define one of multiple spermatogenic events. One other hand, several specific genes such as POU5F1 (also known as OCT3/4) (Pesce et al., 1998), UCHL1 (also known as PGP9.5) (Kon et al., 1999) and PLZF1 (also known as ZBTB16) (Barna et al., 2002; Costoya et al., 2004), Ret and GFRα1 (Meng et al., 2000) has been described to be expressed in undifferentiated spermatogonia, some of which play a critical role for the establishment of stem cell pool and the maintenance of stem cell activity. However, these markers are also expressed gonocytes, and thus cannot distinguish the characteristics of two populations with regard to their functional differences in the early neonatal testis. One approach to distinguishing and characterizing a stage of germ cells in a mixed germ cell population is to identify a stage-specific glycosylation event. The distribution of carbohydrate chain on the cytoplasm and the surface of developing germ cells, which are corresponding to primordial germ cells (PGCs) (Fazel et al., 1990; Fazel et al., 1987), gonocytes (Nagano et al., 1999) and spermatogenic cells (Koshimizu et al., 1995), are associated with their behavioral patterns as the migration toward the basement membrane of

the seminiferous tubule and the differentiation to further spermatogenic stage. Additionally, glycosylation on the gonocytes can be used as a stage-specific marker in early spermatogenic differentiation (Nagano et al., 1999). Therefore, the definition of glycosylation pattern on the early developing testis may provide critical information to understand the functional differences in heterogeneous germ cell population and the mechanisms of initial gonocyte differentiation.

The study on the initial differentiation of germ cells has limited, because of difficulties in the precise identification of characteristics of the heterogeneous cell population in the testis. Therefore, the establishment of *in vitro* culture system and purifying a type of gem cells can provide a useful tool to explore the characteristics and development of germ cells. The in vitro culture technique has been established in various animal species such as mouse (Kanatsu-Shinohara et al., 2005; Kubota et al., 2004; Nagano et al., 1998; 2003), rat (Hamra et al., 2005), hamster (Kanatsu-Shinohara et al., 2008a) and rabbit (Kubota et al., 2011). In the domestic animal species, however, the culture systems have not been available for germ cells of domestic animal due to lack of knowledge with regarding to the maintenance of germ cell properties *in vitro* condition.

Cell surface molecules such as glycoproteins and carbohydrate chains in germ cells development are essential for regulating the spermatogenesis and the maintenance of self-renewal in mammalian species. Recent studies have revealed that adhesion proteins, such as β1- and α6-integrin, are present on the surface of mouse SSCs, which support the long-term proliferation of SSCs in culture (Kanatsu-Shinohara et al., 2005; Shinohara et al., 1999) and play critical roles in the reconstruction of the stem cell niche after transplantation into immunodeficient mouse testis (Kanatsu-Shinohara et al., 2008b). Therefore, the adhesion molecules seem to be associated with their survival and proliferation both *in vitro* and *in vivo*.

In addition, carbohydrate moieties of glycoprotein as known *a-N-acetylgalactosamine* (GalNAc) residues in spermatogenesis have been used a useful stage specific marker. The distribution of these glycan epitopes closely associated with the behavioral pattern of developing germ cells, and they are an essential factor for survival of germ cells. However, the functional roles of these glycan epitopes are little known in the development of germ cells in the mammalian species. The aim of this study is to extend our knowledge of the distribution and physiological feature of glycan epitopes on germ cell development *in vitro* and *in vivo*.

CHAPTER 2

Characterization and identification of gonocytes by the binding affinity of lectin *Dolichos biflorus agglutinin* in neonatal mouse testis

2.1 Introduction

Spermatogenesis is a unique reproductive system and can produce spermatozoa through the complex developmental cascade during the postnatal life of male germ cell development. Spermatogonial stem cells (SSCs) that are based on spermatogenesis were derived from gonocytes, which give rise to the first wave of spermatogenesis in the early postnatal stage (de Rooij and Russell, 2000; Yoshida et al., 2006). In the mice, gonocytes originate from primordial germ cells (PGCs) at around 13.5 post coitum (dpc) and actively proliferate until 16.5 dpc. At this point, they enter the quiescent stage, at which gonocytes are arrested in the G0/G1 phase of the cell cycle (Hilscher and Makoski, 1968). After birth, subpopulations of these cells reinitiate mitotic division and are translocated the basement membrane of the seminiferous tubules (Clermont and Perey, 1957b; McLean et al., 2003). Since germ cells firstly acquire stem cell properties and rapidly expand their population (Spradling et al., 2001), these behavioral features of germ cells are critically important to establish spermatogenesis (McGuinness and Orth, 1992a, b). Altogether these studies indicate that gonocytes in developing testis exhibit a dynamic change, the regulatory mechanism of their physiological change during the postnatal phase of gonocytes has not yet been known. Therefore, the careful characterization of gonocytes is necessary to understand how to regulate the reinitiation of cell proliferation and what physiologically difference between gonocytes and spermatogonia.

Cell surface glycan epitopes has been known to play important roles in cell differentiation (Damjanov, 1987), cell recognition and adhesion (Sharon and Lis, 1989) and cell to cell interaction (Wassarman, 1989). These surface glycans consist of the basement membrane of testicular germ cells has been investigated in the control of spermatogenesis, including spermatogonial proliferation (Ertl and Wrobel, 1992) and meiosis (Koshimizu et al., 1993). In addition, glycan epitopes that compose of cell surface of the developing germ cells exhibit a stage-specific pattern, indicating that the physiological features of these molecules closely related to the transition of their characteristics in the development of germ cells (Muramatsu, 1988a, b). The distribution of glycan epitope in germ cell development of mammalian species has been studied using lectin-immunohistochemistry. Lectins have been used to identify, characterize, and purify objective population on the basis of the presentation of specific carbohydrate groups on the cell surface. The distribution of lectin binding in the testis has been studied in human (Arenas et al., 1998), rodents (Martinez-Menarguez et al., 1992) and domestic animals (Arya and Vanha-Perttula, 1985; Ertl and Wrobel, 1992; Goel et al., 2007). In this study, we describe the binding affinity of the Lectin *Dolichos biflorus agglutinin* (DBA), which specifically recognizes GalNAc residues, toward testicular germ cells including gonocytes and spermatogonia in mouse testis.

2.2 Materials and Methods

2.2.1 Preparation of tissue sections on mice and porcine testis

All animals used in this study were handled according to the guideline of the Institutional Animal Care and Use Committee of Kyoto University. Mice testes were collected from 0.5 dpp to 8-week-old mice (C57BL/6J). On other hand, porcine testes were collected from crossbred piglets (Landrace×Large White Yorkshire×Duroc) aged 3 days after

birth from a local farm as described previously (Goel et al., 2009). The testis samples of both mouse and pig were immediately fixed after collection for immunohistochemistry in both Bouin's and 4% (w/v) paraformaldehyde (PFA) in PBS fixative solution for 4 h and overnight at 4℃, respectively. For paraffin embedding, the testes were firstly proceeded the dehydration step with a series of alcohols and were washed 3 times with xylene. Finally, specimens were embedded in paraffin and were sectioned into 6 ㎛ thick slices. These samples were stored at 4℃ until use.

2.2.2 Immunohistochemical analysis of developing testes by using the heat-mediated antigen retrieval technique

Paraffin sections were serially dewaxed, rehydrated in graded alcohols and washed with TBS (50mM Tris-base; 150mM NaCl). To achieve antigen retrieval (Shi et al., 1991), testis sections were followed by a heat-mediated antigen retrieval process in 10 mM citrate buffer (pH 6.0) by autoclaving, and washed with TBS. After antigen retrieval, samples were then permeabilized for 15 min with 5% (v/v) Triton X-100 in TBS, incubated with 3% (v/v) hydrogen peroxide in TBS for 10 min to block endogenous peroxidases and washed with 0.05 % (v/v) Tween 20 in TBS (TBS-T). Testis sections were incubated in 10% (w/v) normal goat serum in TBS for 1 h at 37℃ and were incubated in biotinylated DBA (1:300, Vector Laboratories, Burlingame, CA, USA) for overnight at 4℃ in a moist chamber. After incubation with DBA, samples were rinsed with TBS-T three times. Avidin-biotin complex (ABC) methods (Vectastain ABC Kit, Vector Laboratories, Irvine, CA, USA) were used for the optimal sensitivity against DBA binding according to manufacturer's instructions before use and rinsed with TBS-T three times. To visualize DBA, samples were developed for 3-5

min with 3, 3'- diaminobenzidine tetrahydrochloride (DAB) (Dojindo, Kumamoto, Japan) as chromogen and 0.01% (v/v) H_2O_2 in TBS-T, counterstained with hematoxylin, and rinsed with TBS. After dehydration, sections were mounted with xylene-based Permount mounting medium (Fisher Scientific Company, Pittsburgh, PA, USA), and observed under a microscope (Olympus BX 50, Tokyo, Japan).

2.2.3 Immunolocalization of germ cell-specific markers in developing germ cells

Germ cell-specific marker UCHL1 used to determine a germ cell population on developing testis. The expressions of germ cell-specific markers were detected using conventional immunohistochemistry as described above. Briefly, paraffin embedded samples were dewaxed, rehydrated, performed antigen-retrieval procedure, and permeabilized. Samples were incubated to block the non-specific binding with 5% (v/v) bovine serum albumin (BSA; Sigma, Louis, Mo, USA) in TBS-T for 1 h at 37℃, followed by incubation with primary antibody (anti-UCHL1; 1:1000; Biomol, Exeter, UK) for overnight at 4℃ and rinsed several times with TBS-T. The primary antibodies recognized their corresponding secondary antibody as an anti-rabbit IgG antibody conjugated with Alexa 546 (1:500; Molecular Probes, Eugene, Oregon, USA) for 1 h at 37℃, rinsed three times with TBS-T. The samples were counter-stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, mounted with 50 % glycerol in TBS and observed under an immune-fluorescence microscope (Olympus BX 50, Tokyo, Japan).

2.3 Results

2.3.1 Improvement of DBA signals on porcine gonocytes using the heat-mediated antigen

retrieval (HMAR) technique

The heat-mediated antigen retrieval (HMAR) is a heat-based antigen unmasking technique that can be used prior to immunohistochemical staining on formalin-fixed paraffin-embedded tissue sections (Shi et al., 1991). To restore the affinity of glycan epitopes to DBA on the surface of germ cells, the HMAR technique was performed in present study. Immunohistochemical staining showed that the DBA signals and the specific affinity of anti-POU5F1 against gonocytes were weekly detected on the neonatal porcine testis without the HMAR technique (Fig. 2.1A and B). The signals of DBA binding were observed the partial pattern on the surface of gonocytes in the seminiferous tubule (Fig. 2.1A, arrows). Also, the specific binding affinity of anti-POU5F1 on gonocytes was weekly detected in the neonatal porcine testes (Fig. 2.1B, arrows). Using HMAR technique, however, DBA signals were intensely detected on gonocytes of the neonatal testis (Fig. 2.1C) with compared to without HMAR treatment (Fig. 2.1A). DBA signals were observed on the cell surface as well as the cytoplasm of gonocytes (Fig. 2.1C, arrows). DBA binding were occasionally observed on the interstitial tissue in the testes. Expectedly, the binding affinity of anti-POU5F1 was effectively improved using the HMAR treatment (Fig. 2.1D, arrows).

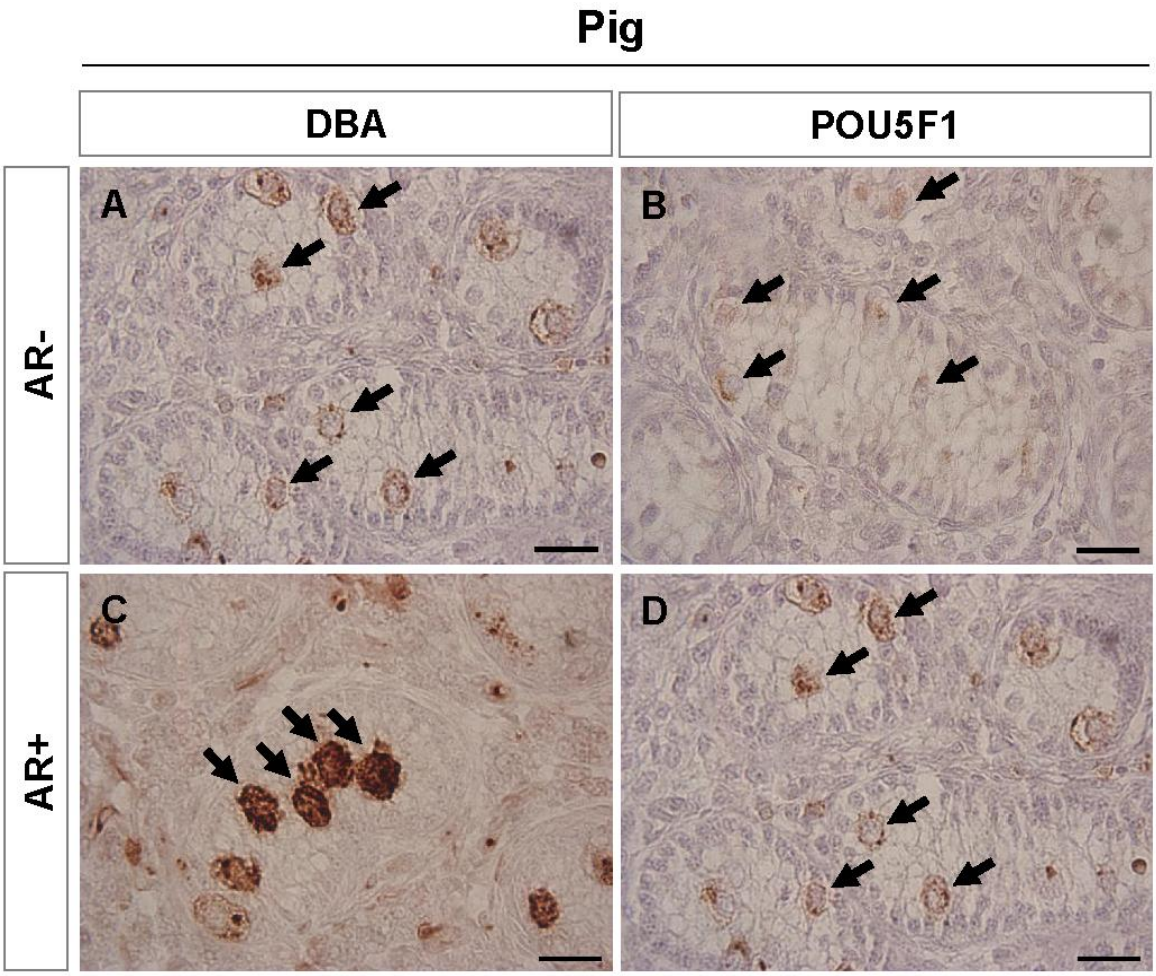

Figure 2.1 The specific affinity of lectin DBA and anti-POU5F1 reactivity to gonocytes by the heat-mediated antigen retrieval technique (HMAR) in neonatal porcine testis. (A-B) The specific affinities of DBA binding and germ cell-specific antibody POU5F1 to germ cells were examined in neonatal testes without the HMAR treatment. The DBA (A) and POU5F1 (B) signals were detected on the procaine gonocytes, but show weak signals. (C-D) DBA binding and anti-POU5F1 reactivity was examined with the HMAR treatment on the neonatal porcine testes. The DBA signals (C) were intensely detected with applying the HMAR treatment, and the POU5F1 reactivity (D) increased on the gonocytes. Arrows that represented gonocytes stained with DBA (A and C) or POU5F1(B and D) AR-, HMAR non- treatment; AR+, HMAR treatment. Bar = 20㎛.

2.3.2 Identification of the binding affinity of DBA on germ cells in the developing mouse testis by the HMAR treatment

DBA signals were not detected on the testis section both 1 dpp (Fig. 2.2A) and 8-week-

old (Fig. 2.2B) without HMAR treatment. Using HMAR treatment, however, DBA signals were detected on germ cell population in both neonatal testis at 1 dpp (Fig. 2.2C) and adult testis at 8-week-old (Fig. 2.2D). In neonatal testis, gonocytes that located at the central part (Fig. 2.2C, arrow heads) and periphery (Fig. 2.2C, arrows) of seminiferous tubules were stained with the lectin DBA, while a population of somatic cells and interstitial cells do not stained. Additionally, spermatogenic populations of the differentiating stage were stained with DBA in adult testis at 8-week-old (Fig. 2.2D, brown color), but undifferentiating spermatogonia that were contacted to the basement membrane of the seminiferous tubule were not stained (Fig. 2.2D, arrows). These results indicate that presentation of GalNAc residues on mouse germ cells can be detected by using the HMAR technique.

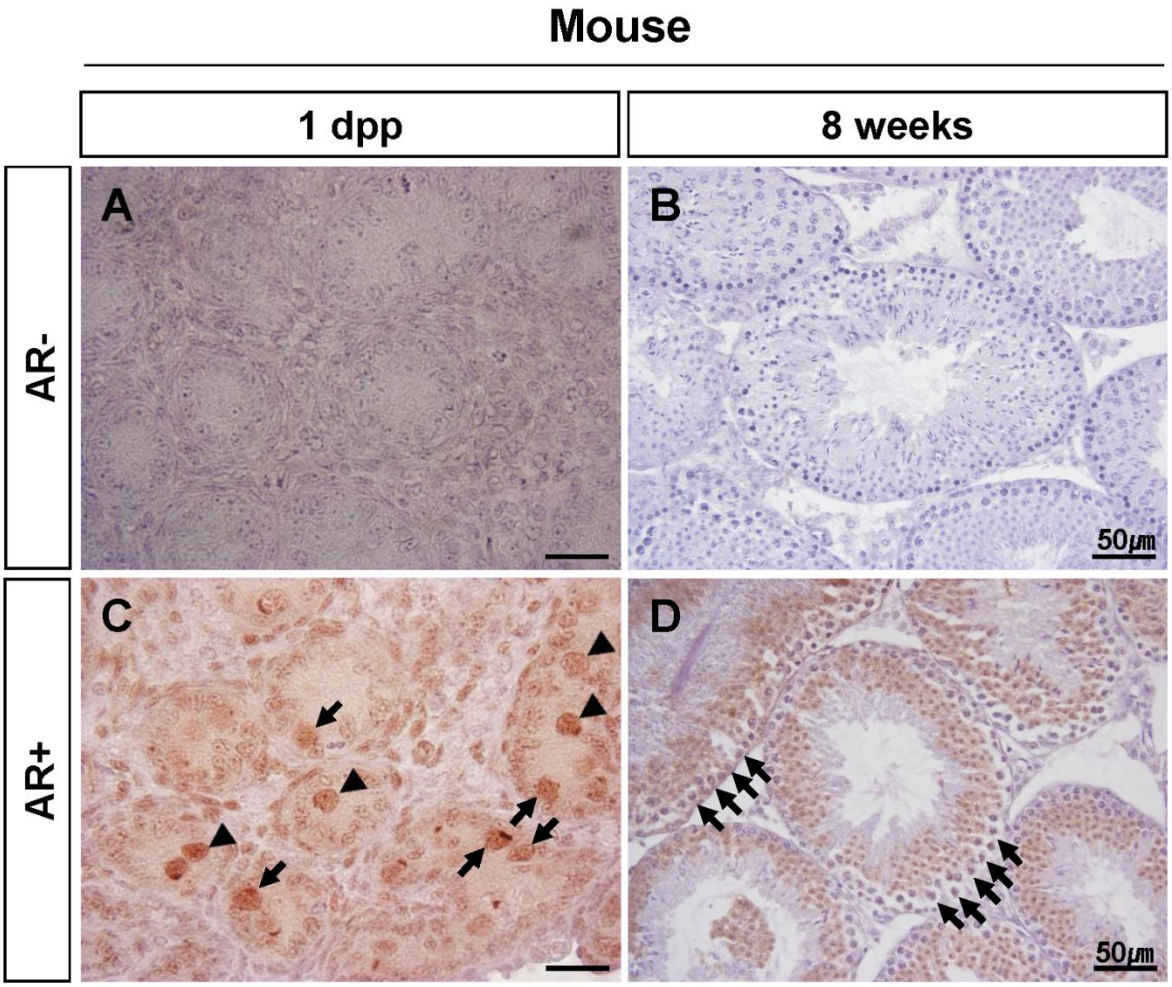

Figure 2.2 The determination of DBA binding affinity to glycan epitopes using the heat-mediated antigen retrieval technique (HMAR) in mouse testis. (A-B) The testis samples subjected the lectin-immunohistochemistry without the HMAR treatment in the neonatal and adult mouse testis. The DBA signals were not detected both the neonatal (A) and adult testis (B) Testis sections counter-stained with Hematoxylin. (C-D) Lectin-immunohistochemistry was performed by using the HMAR treatment in the mouse testis. The DBA signals were detected on germ cell populations in the neonatal (C) and adult mouse testis (D). Arrow heads show the germ cells that located on the central part of seminiferous tubules (C), and arrows that indicate germ cells, which were translocated tothe basement membrane of seminiferous tubules (C and D). AR-, HMAR non- treatment; AR+, HMAR treatment. Bar = 20μm.

2.3.3 Differential distribution of DBA signals in developing testis

To examine the distribution of DBA signals in testis at the different age of 1 dpp, 7 dpp

and 8 weeks, testis samples were stained with the lectin DBA (Fig. 2.3A, B and C) and a germ cell-specific marker UCHL1 (Fig. 2.3D, E and F). In the neonate stage, DBA signals were detected on gonocytes at 1 dpp (Fig. 2.1A, arrows), but the signals disappeared and partially stained as condensed dots at 7 dpp (Fig. 2.3B, arrows). In contrast, the expression of UCHL1 was strongly observed at both 1 dpp (Fig. 2.1D, arrows) and 7 dpp (Fig. 2.1E, arrows), and the numbers of UCHL1-positive gem cells were markedly increased at 7dpp. Also, they were translocated from the central part to the periphery region of the seminiferous tubule (Fig. 2.1E), even though some of gonocytes at 1 dpp already had migrated toward the basement membrane of seminiferous tubule. On the testes of 8-week-old (Fig. 2.3C and F), DBA signals (Fig. 2.3C) were only detected on differentiating spermatogonia, while UCHL1 (Fig. 2.3F) was only expressed on undifferentiated spermatogonia.

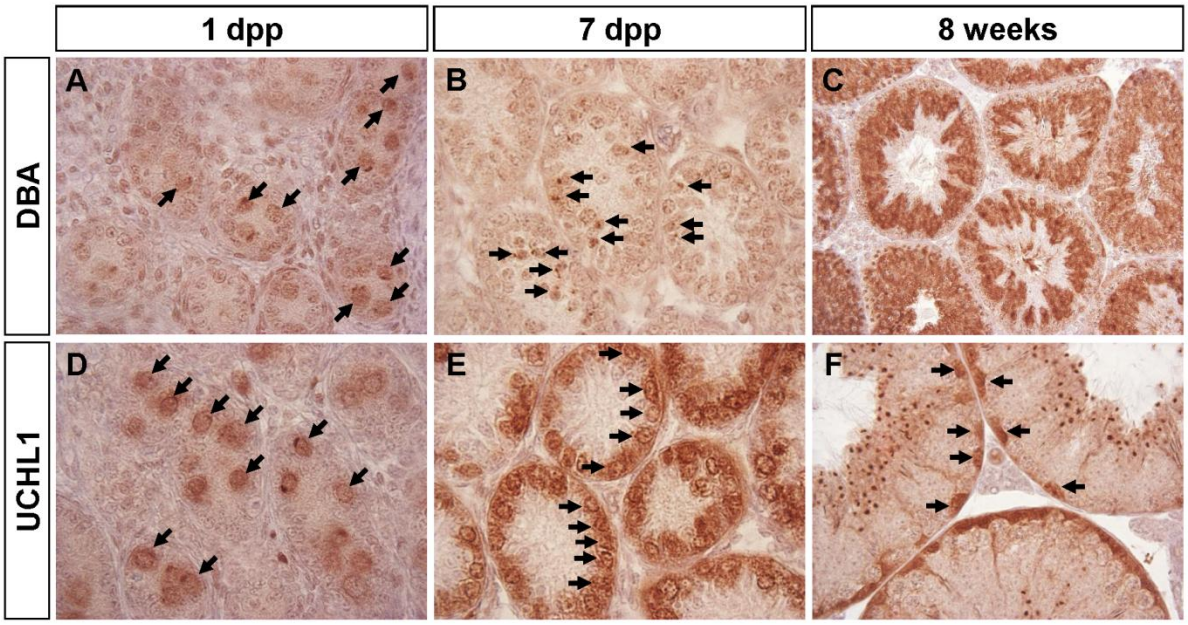

Figure 2.3 Comparison between DBA-binding affinity and UCHL1 expression during the development of germ cells in mouse testis. (A-F) Testes samples at the different ages of 1 dpp (A and D), 7 dpp (B and E) and 8-week-old (C and F) were stained with DBA (A, B and C) and UCHL1 (D,

E and F). (A and D) Gonocytes that are present in the mouse testis of 1 dpp were stained with both DBA (A, arrows) and anti-UCHL1 (D, arrows). At 7 dpp, DBA signals (B) were disappeared and partially stained, while the UCHL1 expression (E) was continued in cell population of the seminiferous tubules. On the adult testis (C and F), the differences in the DBA signals (C) and UCHL1 (F, arrows) expression were observed on the seminiferous epithelium. DBA signals (C) were detected on the most of spermatogonial population, whereas the UCHL1 expression (F) only detected on the periphery region of seminiferous epithelium.

2.4 Discussion

Carbohydrate residues linked to proteins with O- or N-linked saccharides are known to be a stage-specific pattern on cell surface and have an important role for germ cell development (Akama et al., 2002; Kanai et al., 1989). The presentation of carbohydrate residues depends on developing gem cells in pig (Goel et al., 2007) and cattle (Ertl and Wrobel, 1992), suggesting that the detection of GalNAc residues on germ cells is useful for understanding the functional difference of heterogeneous population of germ cells such as gonocytes and type A spermatogonia in postnatal mice testis. In this study, the specific affinity of DBA against glycan epitopes on gonocytes both porcine and mouse testis could be improved by using the HMAR technique (Fig. 2.1 and 2.2). In the mouse testis, DBA signals could observe on germ cells using the HMAR technique, whereas the testis samples in the non-HMAR treatment were not showed the DBA signals (Fig. 2.2). In addition, the DBA signals showed the differential distribution on the developing mouse testis (Fig. 2.3).

In the mouse testis, the DBA-binding has been seen in differentiating spermatogonia such as spermatocytes and spermatid in the stage VII of spermatogenic cycle (Arya and Vanha-Perttula, 1986; Lohr et al., 2010), but not in Sertoli cell and the inter-tubular interstitium (Lohr et al., 2010). In the previous reports, however, DBA does not have the binding affinity in the earlier stage of germ cells such as gonocytes and spermatogonia

(Izadyar et al., 2002; Nagano et al., 1998; Zhang et al., 2008). The inconsistent results with regarding to the DBA binding affinity on mouse germ cells have not been clarified yet. However, it has been known that the loss of binding epitopes is caused by the formalin-induced cross-linking of proteins on the tissue processing (Shi et al., 1991). In addition, the distribution of ligands can be modified by the duration of fixation and the type of fixative, and the use of organic solvents such as methanol, xylene and acetone are caused by the loss of ligands and glycolipids (Schwarz and Futerman, 1997). The HMAR technique has been known as useful tool to rescue the binding epitopes in the immunochemical staining (Shi et al., 2007). In our previous reports, porcine gonocytes show the DBA-binding by using a usual fixation process and the procedure for lectin-histochemical staining (Goel et al., 2007), but the signal of DBA became more clearer by using the HMAR technique (Fig. 2.1). These observations can be considered that the affinity of DBA binding to germ cells depends on the tissue processing and the procedure for the lectin-immunohistochemical staining. Although the principle on the rescue of the carbohydrate structure were not obvious in present study, the HMAR technique improve the affinity of DBA binding to glycan epitopes, and is recognized a useful tool to define the dynamical modification of the distribution of GalNAc residues on gonocytes.

The specific binding affinity of DBA was strongly detected on gonocytes (Fig. 2.2), and the distribution of DBA signals on germ cells was changed in the different age of testis (Fig. 2.3). Lectin DBA that specifically recognizes GalNAc residues can be used to identify primordial germ cells (PGCs) (Takagi et al., 1997; Wrobel and Suss, 1998), gonocytes (Goel et al., 2007) and type A spermatogonia (Izadyar et al., 2002) in domestic animal species. The DBA signals show the differential pattern during the transition of gonocytes to the basement membrane, which are coincident with the period as the onset of spermatogenesis, in the early

developing testis of pig (Goel et al., 2007) and cattle (Herrid et al., 2007). These molecular characteristics are indicating that the differential distribution of DBA signals may arise from the change of glycosylation pattern on gonocytes, suggesting that the specific affinity of DBA on gonocytes may be a common feature in the mammalian species, and their affinities were altered following the differentiation process of germ cells.

CHAPTER 3

Modification of cell surface glycan epitopes associated with the initial differentiation process of primitive male germ cells in neonatal mouse testes

3.1 Introduction

Gonocytes are located on the central part of the seminiferous tubules in postnatal testis (Clermont & Perey 1957) and are under a mitotically quiescent state. During the early postnatal period, gonocytes resume mitotic division and were relocated toward the basement membrane of the seminiferous tubule for the onset of spermatogenic process (Clermont & Perey 1957, Huckins & Clermont 1968). Eventually, gonocytes give rise to spermatogenic cell populations, which are referred to as type A spermatogonia. The term of type A spermatogonia contains two types of spermatogonia, undifferentiated spermatogonia (Aund), which correspond to Asingle, Apaired and Aaligned spermatogonia (de Rooij & Russell 2000) and differentiating type A spermatogonia, which involve A1 to A4 and Intermediate (In) spermatogonia. Additionally, Aund spermatogonia consist of a heterogeneous population, which exhibits a stage-specific expression pattern of the germ cell specific proteins such as Nanos2 and Nanos3 (Suzuki et al. 2009). In neonatal testis, however, the initial differentiation processes of gonocytes to initiate spermatogenesis are not fully understood. Some reports described that gonocytes produce prospermatogonia and differentiate to type A spermatogonia (Hilscher et al. 1974, Bellve et al. 1977), and the others reported that gonocytes differentiate directly to type A spermatogonia (de Rooij 1998, de Rooij 2001). Recently, the gonocytes and/or A1 spermatogonia in early neonatal testis can directly differentiate to type A4 spermatogonia by skipping the sequential differentiation steps

(Drumond et al. 2011). These studies were defined cell characteristics by the morphological features of developing germ cells, such as diameter and the shape of nucleus, the construction shape of nucleolus and the number of heterochromatin spots, through a high-resolution microscopic approach. However, the molecular and physiological characteristics of gonocytes on differentiation processes remain unknown. It is mainly due to the difficulty of precise definition of differentiation and development of gonocytes. Therefore, specific markers derived from gonocytes are necessary for understanding germ cell development after birth.

Cell surface markers can provide a powerful tool for investigating of a heterogeneous population of gonocytes during early spermatogenesis. Several markers, such as EE2 antigen (Tanaka et al. 1997), THY1 (Ryu et al. 2004) and EpCAM (Anderson et al. 1999), have been used for the characterization of germ cells in mice testis. However, these markers broadly express in differentiating gonocytes and there are no markers to define the differentiation processes to spermatogonia. Meanwhile, the patterns of carbohydrate residues on the surface of germ cells are a stage-specific and closely related to cell-to-cell interaction and the cell differentiation in the development of germ cells (Muramatsu 1988). Therefore, the terminal carbohydrate residues might be used as stage-specific markers for the definition of a differentiation step of gonocytes during the early developing period in the postnatal testis.

Lectins can recognize specific carbohydrate residues on the cell surface widely used to investigate physiological changes of developing germ cells. Previous studies has been observed that a lectin, Dolichos biflorus agglutinin (DBA), recognizes α-N-acetylgalactosamine (GalNAc) residues (Piller et al. 1990) and the residues are found on the surface of germ cells in mammalian species such as cattle (Ertl & Wrobel 1992), pig (Goel et al. 2007) and horse (Verini-Supplizi et al. 2000). Although the role of terminal glycans on the development and differentiation of germ cells has been little known, the affinity of

carbohydrate residues to DBA depend on the type of germ cells in developing testis. Our previous study described that the distribution of terminal glycan residues on gonocytes by using DBA could be identified and characterized in pig (Goel et al. 2007) and cattle (Kim et al. 2013), suggesting that the distribution of glycan residues may be associated with the differentiation process of germ cells. Therefore, the detecting the glycan epitopes by DBA in developing germ cells can provide a useful tool to identify and characterize the physiological difference in the distinct germ cell populations.

In the present study, we examined whether the affinity of DBA to GalNAc residues on the surface of gonocytes can be used for the identification of differentiating gonocytes during earlier development of germ cells in postnatal mouse testis. Furthermore, possible role of GalNAc residues on gonocytes during the initial differentiation process of gonocytes was investigated.

3.2 Materials and Methods

3.2.1 Preparation of tissue sections from the porcine and mice testes

All animals used in this study were handled according to the guideline of the Institutional Animal Care and Use Committee of Kyoto University. Mice testes were collected from 0.5 dpp to 8-week-old mice (C57BL/6J). Porcine testes were collected from crossbred piglets (Landrace×Large White Yorkshire×Duroc) aged 3 days from a local farm as described previously (Goel et al. 2009). The testis samples from mice and pig were immediately fixed with Bouin's and 4% (w/v) paraformaldehyde (PFA) in PBS fixative solution for 4 h and overnight at 4℃, respectively. For paraffin embedding, the testes were firstly proceeded the dehydration step with a series of alcohols and were washed 3 times with xylene. Finally, specimens were embedded in paraffin and were sectioned into 6 ㎛ thick slices. These

samples were stored at 4℃ until use.

3.2.2 Detection of DBA affinity and germ cell-specific markers on germ cells

Immunohistochemical analysis was performed using a conventional method as previously described by Goel et al. (2007). Briefly, paraffin sections were serially dewaxed, rehydrated in graded alcohols and washed with TBS (50mM Tris-base; 150mM NaCl). Testis sections were followed by a heat-mediated antigen retrieval process in 10 mM citrate buffer (pH 6.0) by autoclaving, and washed with TBS (Shi et al. 1991). Samples were then permeabilized for 15 min with 5 % (v/v) Triton X-100 in TBS, incubated with 3% (v/v) hydrogen peroxide in TBS for 10 min to block endogenous peroxidases and washed with 0.05 % (v/v) Tween 20 in TBS (TBS-T). Testis sections were incubated in 10 % (w/v) normal goat serum in TBS for 1 h at 37 ℃ and were incubated in biotinylated DBA (1:300, Vector Laboratories, Burlingame, CA, USA), anti-DDX4 (1:300; Chemicon, Temecula, CA, USA), anti-POU5F1 (1:50; C-10, Santa Cruz Biotechnology, CA, USA) and anti-UCHL1 (1:1000; Biomol, Exeter, UK) antibodies for overnight at 4℃ in a moist chamber, rinsed several times with TBS-T. After incubation with primary antibodies, samples were rinsed with TBS-T three times, incubated for germ cell-specific markers with the corresponding biotin-conjugated secondary antibodies, i.e., swine anti-rabbit IgG (1:300, DAKO, Carpinteria, CA, USA) for 1 h at room temperature and rinsed several times with TBS-T. Avidin-biotin complex (ABC) methods (Vectastain ABC Kit, Vector Laboratories, Irvine, CA, USA) were used for the optimal sensitivity against the DBA-binding and germ cell-specific markers according to manufacturer's instructions before use and rinsed with TBS-T three times. To visualize DBA and germ cell-specific markers, samples were developed for 3-5 min with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Dojindo, Kumamoto, Japan) as chromogen and

0.01% (v/v) H2O2 in TBS-T, counterstained with hematoxylin, and rinsed with PBS. After dehydration, sections were mounted with xylene-based Permount mounting medium (Fisher Scientific Company, Pittsburgh, PA, USA), and observed under a microscope (Olympus BX 50, Tokyo, Japan).

3.2.3 Immunolocalization of DBA and stage-specific markers in differenting germ cells

To analyze differential binding affinity of DBA on developing germ cells, double-immunofluorescence staining was performed by using DBA, germ cell-specific markers (UCHL1; 1:1000 and anti-DDX4; 1:300) and a premeiotic marker STRA8 (1:250; Abcam, Cambridge, MA, USA). Briefly, testis sections were dewaxed, rehydrated, performed antigen-retrieval procedure, and permeabilized as described above. Testis sections were blocked with 10% normal goat serum for the DBA-binding and 5% (v/v) bovine serum albumin (BSA; Sigma, Louis, Mo, USA) for DBA and antibodies staining in TBS-T for 1 h at 37 ℃, followed by incubation with primary antibodies (DBA, anti-UCHL1 and anti-DDX4) for overnight at 4 ℃ and rinsed several times with TBS-T. Samples were incubated with FITC-conjugated anti-streptavidin (1:300, DAKO, Carpinteria, CA, USA) and Alexa Fluor 546-labeled anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR, USA) for 1h at 37 ℃, counterstained with Hoechst 33342 (1:1000, Sigma) for 5 min, washed seven times with TBS-T, mounted with 50 % (v/v) glycerol in PBS, and observed under microscope (Olympus BX 50).

3.2.4 Statistical analysis

All data are presented as mean ± S.E.M. Comparisons of multiple group were analyzed

using the one-way analysis of variance (ANOVA) and two-way ANOVA followed by Tukey's and Bonferroni post-hoc pairwise comparison at each group. A level of $p<0.05$ was considered statistically significances. Statistical analysis of this study was performed using StatView (ver. 5.01, SAS Institute, CA, USA) and GraphPad Prism (ver. 4.00, GraphPad software, CA, USA).

3.3 Results

3.3.1 Dynamic change of DBA affinity to gonocytes during the early developmental stage in mouse testis

A dynamic state of the DBA binding to gonocytes at the postnatal stage of the mouse testis was examined. The DBA binding was detected on the cell surface and cytoplasm, but the number of DBA-positive cells was decreased with the progression of the early developmental stage, which corresponds to 0.5 dpp to 5 dpp (Fig 3.1). At 5 dpp in the testis, DBA signals were observed as condensed dots on gonocyte cytoplasm (Fig. 3.1, white arrows). The testes samples were subjected immunohistochemistry to determine the affinity of DBA binding (Fig. 3.2A and E) and the expression of germ cell-specific markers UCHL1 (Fig. 2B and F), DDX4 (Fig. 3.C and G) and POU5F1 (Fig. 3.2 D and H) on the germ cells. Gonocytes that are residue in testes of 0.5 dpp stained with DBA (Fig. 3.2A, arrows) and germ cell-specific markers (Fig. 3.2B, C and D). At 3 dpp in the testis, however, the DBA-binding (Fig. 3.2E, arrows) were mostly disappeared from gonocytes, while germ cell markers UCHL1 (Fig. 3.2F), DDX4 (Fig. 3.2G) and POU5F1 (Fig. 3.2H) were observed on the gonocytes. At this stage, a few numbers of gonocytes were still remained at the central region of the seminiferous tubules, but most cells were translocated to the basement membrane of the seminiferous tubules (Fig 3.2).

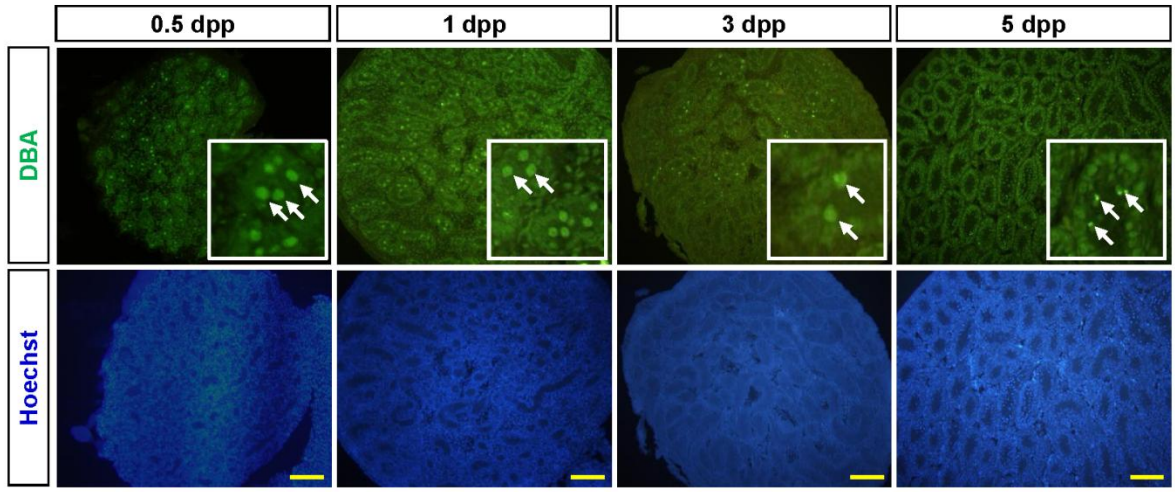

Figure 3.1 Lectin-histochemistry using the lectin DBA during the earlier developmental periods of mouse testis. The signals of DBA on gonocytes were broadly detected on the cell surface from 0.5 dpp to 5 dpp, and their binding pattern was changed to condensed form in gonocytes at 5 dpp. White boxes appear magnified images. All samples were stained with Hoechst 33342. Arrows that were appear gonocytes stained with DBA. Bar = 20㎛.

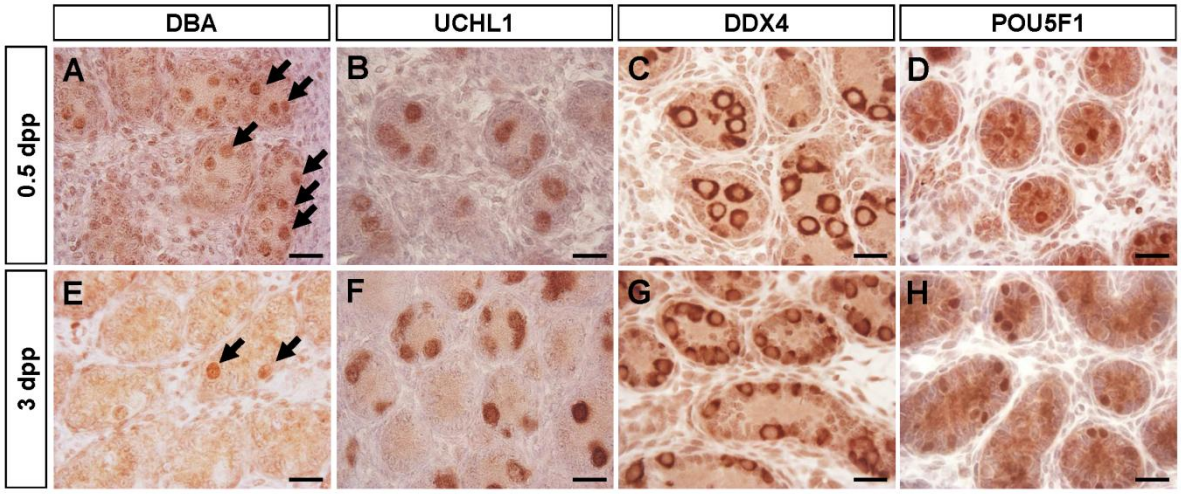

Figure 3.2 Characterization and identification of gonocytes by DBA and germ cell-specific markers during the earlier developmental periods of mouse testis. (A-D) The DBA signals (A) and the expression of germ cell-specific markers UCHL1 (B), DDX4 (C) and POU5F1 (D) were detected on gonocytes at 0.5 dpp. (E-F) At 3 dpp, DBA signals (E) were rarely observed on the gonocytes, while the expressions of germ cell-specific markers UCHL1 (F), DDX4 (G) and POU5F1 (H) were still expressed on gonocytes, which were translocated to the periphery region of the seminiferous tubules (F-H). All samples were counter-stained with Hematoxylin. Arrows that were appear gonocytes stained with DBA. Bar = 20㎛.

3.3.2 Double-immunolocalization of DBA and germ cell-specific marker UCHL1 and DDX4

Testicular sections are double stained with DBA and germ cell-specific markers UCHL1 (Fig. 3.3A) and DDX4 (Fig. 3.3B) to identify gonocytes in the seminiferous tubules. DBA-positive cells that showed the diffused pattern on the cytoplasm and the surface of gonocytes were co-localized with UCHL1 and DDX4 until 3 dpp, but these cells were gradually lost the affinity of the DBA-binding from 2 to 5 dpp (Fig. 3.3 and 3.4). Interestingly, the DBA signals during the same period were changed from the diffused form (Fig. 3.3, green arrows) to the condensed form (Fig. 3.3, white arrows). Also, a part of gonocytes were not shown the affinity of the DBA-binding (Fig. 3.3, red arrows). At 2-3 dpp, therefore, gonocytes were observed as heterogonous populations in the pattern of DBA-binding. The number of DBA-positive cells that were observed diffused form was counted in UCHL1 and DDX4-positive populations. The number of DBA-positive cells was not different statistically between 1 dpp (2.8 cells/tubule) and 2 dpp (2.6 cells/tubule) (Fig. 3.4A). However, the number of these cells was significantly declined around 3 dpp (1.1 cells/tubule, $p<0.05$) and these populations were hardly detected at 5 dpp (0.1 cell / tubule, $p<0.05$) with compared to 1 dpp and 2 dpp, respectively (Fig. 3.4A). The average rates of DBA-positive cells in UCHL1-positive cells showed that 99.9% of UCHL1-positive cells co-localized with DBA at 1 dpp (Fig. 3.4B), whereas 29.2% ($p<0.001$) and 0.7% ($p<0.001$) of UCHL1-positive cells co-localized with DBA at 3 dpp and 5 dpp, respectively (Fig. 3.4B). In contrast, the number of UCHL1- and DDX4-positive cells was not significantly different until 3 dpp, but was significantly increased around 5 dpp ($p<0.05$) with compared to 1 and 2 dpp, respectively (Fig. 3.4A). These result indicate that DBA-binding depend on increasing of age in the neonatal testis of the mice.

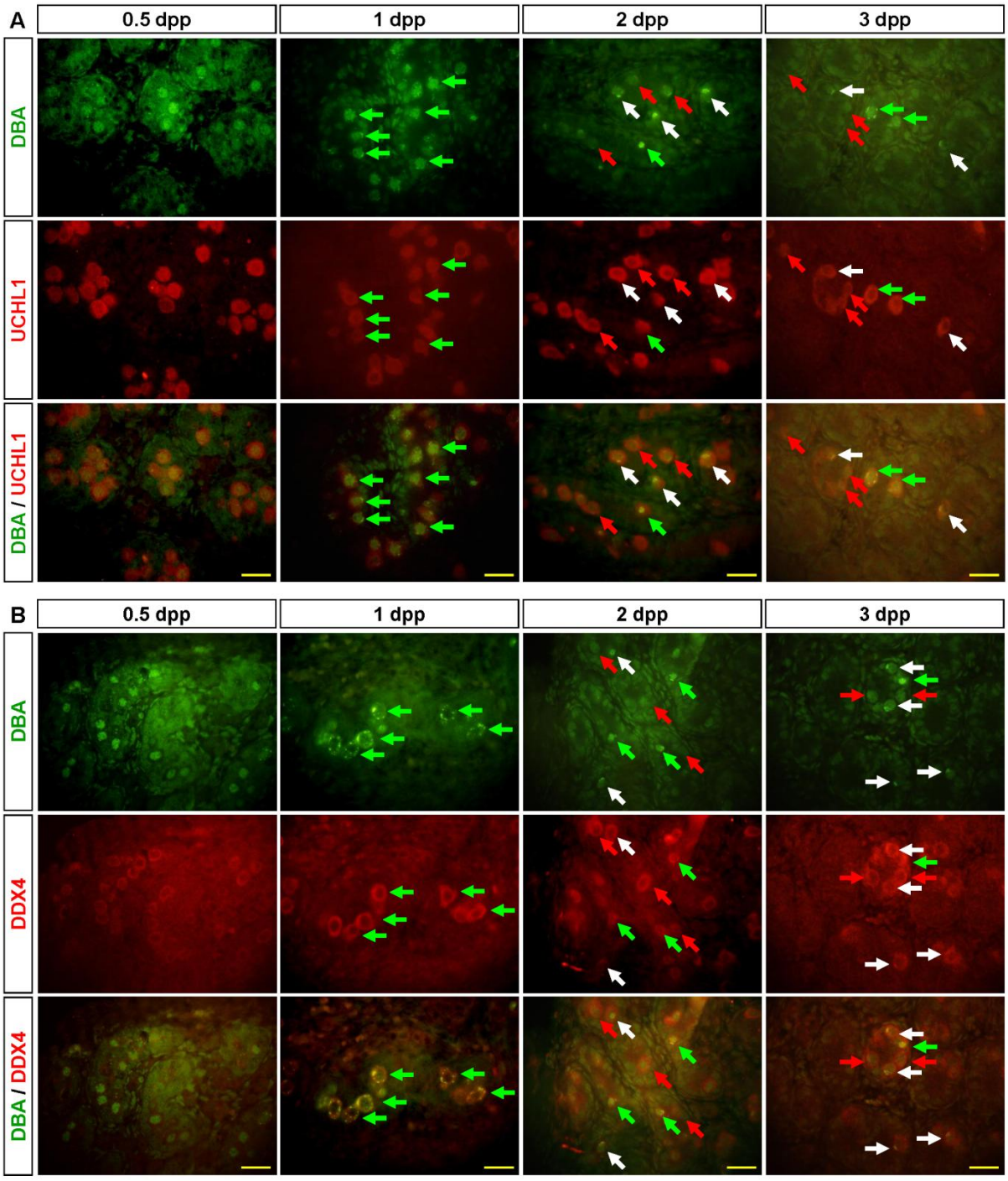

Figure 3.3 Distribution of DBA-positive cells during the early postnatal stage of mouse testis. A and B) DBA was double-stained with UCHL1 (A) or DDX4 (B) from 0.5 dpp to 3 dpp. DBA-positive cells were co-localized with UCHL1 or DDX4, but were rarely observed at 3 dpp. At 2-3 dpp, the DBA-binding patterns in UCHL1 and DDX4-positive cells were detected as diffused form (green arrows), condensed form (white arrow) and no binding signal (red arrow). All images were merged. Bar = 20㎛.

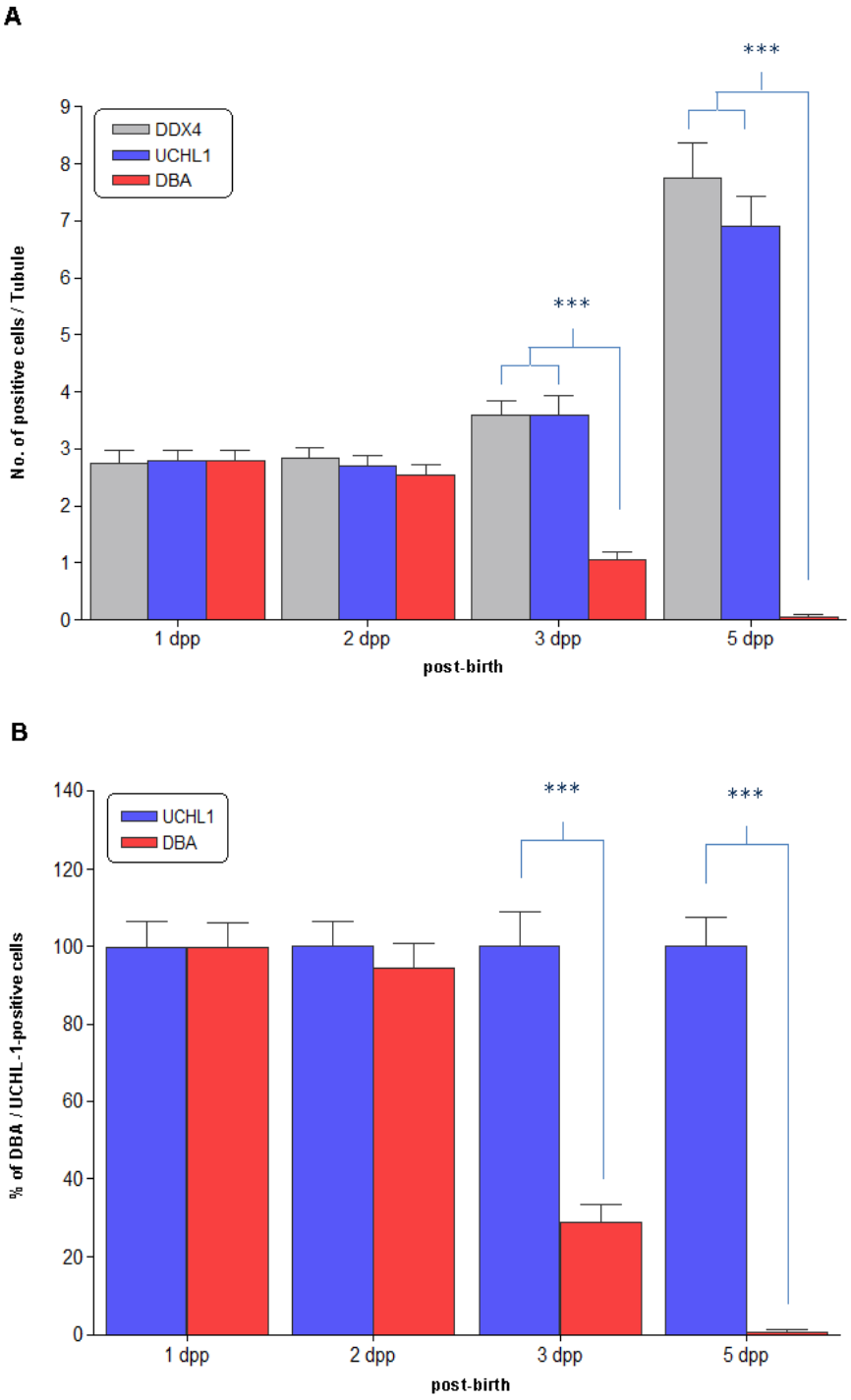

Figure 3.4 The number of UCHL1-, DDX4- and DBA-positive cells during the early postnatal stage of mouse testis. (A) Average numbers of DBA-, UCHL1- and DDX4-positive germ cells estimated in seminiferous tubules. (B) The relative ratios of DBA-positive germ cells against UCHL1-positive germ cells in each developmental stage of mouse testis from 1 dpp to 5 dpp.

*** $p<0.001$ vs. UCHL1 by ANOVA (Bonferroni post-hoc test, respectively)

3.3.3 Fate of gonocytes during the early stage of neonatal testis development

The distribution of DBA binding on gonocytes was strongly and broadly detected at 1 dpp. However, the changing pattern in the affinity of the DBA-binding was observed from 2 dpp (Fig. 3.3) and was completely altered as condensed dots at the perinuclear region of cells at 5 dpp (Fig. 3.5, green arrows). At 5 dpp, these DBA-positive cells were weakly expressed UCHL1 and were located on the periphery of the seminiferous tubules (Fig. 3.5, green arrows). Some of UCHL1-positive cells were not observed DBA signals (Fig. 3.5, white arrows). However, the expression of UCHL1 at 7 dpp was no longer observed in the DBA-positive populations (Fig. 3.5, green arrows). Most UCHL1-positive cells are closely associated with the basement membrane of the seminiferous tubule (Fig. 3.5, white arrows), whereas some of DBA-positive cells were not apart from the basement membrane and were located at inside of the seminiferous tubules from 5 to 7 dpp (Fig. 3.5, red arrows). These observations indicating that the changes of DBA-binding affinity may associate with the translocation of gonocytes in the seminiferous tubules of neonatal testis.

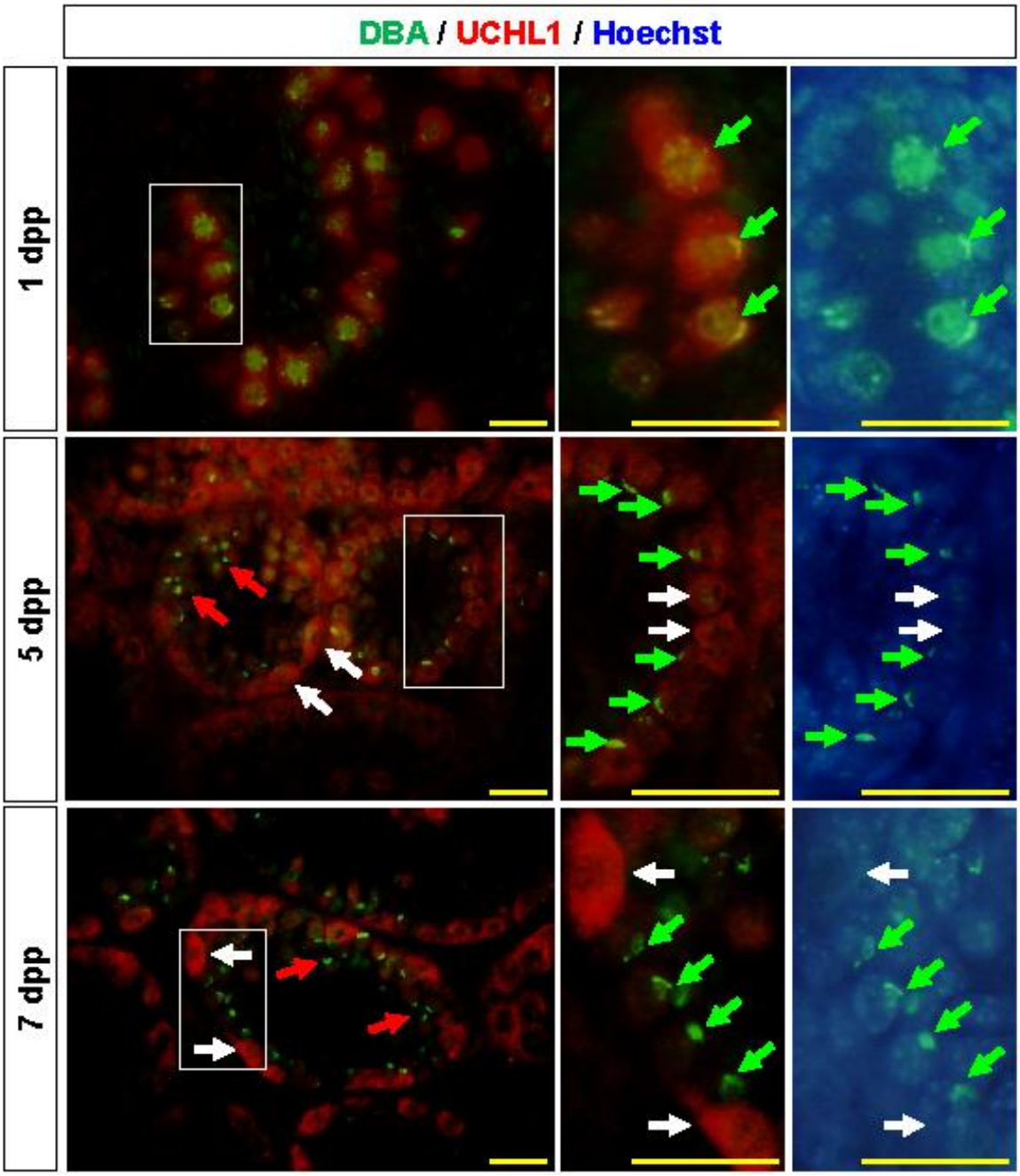

Figure 3.5 Immunohistochemical identification of DBA- and UCHL1-posive cells in postnatal mouse testis at the age of 1, 5 and 7 dpp. Most DBA-positive cells (green) were detected as UCHL1-positive cells (red) at 1 dpp (green arrows). In developing testis at 5 dpp, the affinity of DBA to gonocytes was detected both UCHL1-positive (green arrows) and UCHL1-negative (red arrows) cells. Some gonocytes only expressed UCHL1 without the affinity to DBA (white arrows). A 7 dpp, DBA was only detected in UCHL1-negative cells. White boxes show magnified image. All images were merged with Hoechst 33342-dye staining image. Bar=20㎛.

3.3.4 Determination of the meiotic initiation associated with the distribution of glycan epitopes in developing germ cells

DBA-positive cells inside of the seminiferous tubules at 7 dpp were co-localized with the expression of STRA8 and DDX4 (Fig. 3.6, yellow arrows) and were not detected on the STRA8-negative cells. However, the large numbers of germ cells were observed as DBA-negative and DDX4-positive cells ($DBA^-/DDX4^+$), which closely associated with the basement membrane of the seminiferous tubules (Fig. 3.6, white arrows). Additionally, DBA-positive cells ($DBA^+/DDX4^+/UCHL1^-$) were relatively smaller than DBA-negative ($DBA^-/DDX4^+/UCHL1^+$), which expressed UCHL1 and DDX4 (Fig. 3.5 and 3.6, white arrows).

On other hands, the condensation of DBA signals and UCHL1 expression in adult testis could be detected on the spermatogonial population. The UCHL1 expression was observed on undifferentiated spermatogonia, which was located on the basement membrane of seminiferous tubule (Fig. 3.6C, left panel). Meanwhile, the DBA signals that were detected as condensed form on the perinuclear region of germ cells were observed in the leptotene spermatocytes, which has been known the early meiotic stage of spermatogenesis in adult testis. In contrast with UCHL1 expression, the DBA signals were not observed on undifferentiated spermatogonial populations in adult testis (Fig. 3.6C, right panel).

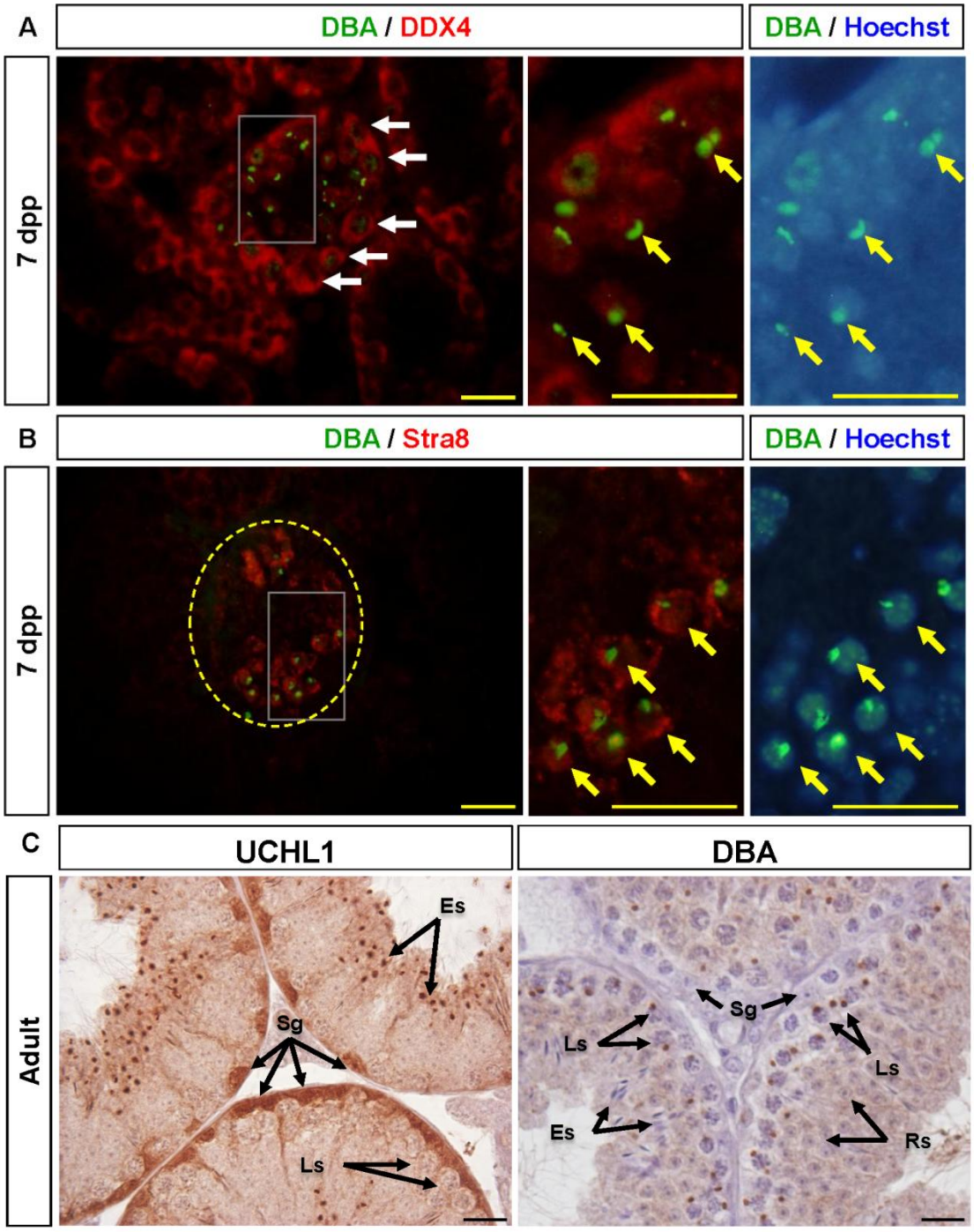

Figure 3.6 Immuno-histochemical detection of DBA signals on the meiotic stage of germ cells at 7 dpp and 8-week-old adult mouse testis. (A) Testis samples were double-stained with DBA and DDX4 at age of 7 dpp. DBA-positive cells were observed in a part of DDX4-positive cells (yellow arrows), which is smaller than DBA-negative and DDX4-positive cells (white arrows) not stained with DBA. Bar=20㎛. (B) The expression of STRA8 protein was determined in testis at 7 dpp. Most DBA-positive cells were expressed STRA8 and were translocated into the inside of the seminiferous tubules

(yellow arrows). Bar=20㎛. (C) Adult testis samples were stained with either UCHL or DBA. The expression of UCHL1 was observed undifferentiated spermatogonia (Sg), which are located on the basement membrane of seminiferous tubules (left panel). The most DBA signals were observed on the perinuclear region of leptotene spermatocytes (Ls), which showed that chromatin of the chromosomes is condensing (right panel). Sg, undifferentiated spermatogonia; Ls, leptotene spermatocytes; Rs, round spermatid; Es, elongated spermatid. Bar=20㎛.

3.4 Discussion

Based on the results described in chapter 2, we hypothesized that the distribution of GalNAc residues on germ cells may be changed depending on the differentiation state of germ cells in mice testis. At the first week after birth, gonocytes resume the mitotic division (McGuinness and Orth, 1992b) and translocate to the basement membrane of the seminiferous tubules (McGuinness and Orth, 1992a). Once gonocytes translocate to the basement membrane, they divide to produce daughter cells (Hilscher et al., 1974) or differentiate into type A spermatogonia after a few cell division (Bellve et al., 1977). Meanwhile gonocytes that are remained on the central part of the seminiferous tubule undergo degeneration (Roosen-Runge and Leik, 1968). Therefore, translocation of gonocytes from the central part of the seminiferous tubules is a critical process for the survival and differentiation of gonocytes. Taken together, in our previous study, the characteristic change of gonocytes during the translocation seems to be associated with cell surface glycan epitopes, which may support the further progression of differentiation process of gonocytes in cattle (Kim et al. 2013). In this study, the loss of DBA-binding affinity in gonocytes occurred at the same time along with the translocation of gonocytes in this study (Fig. 3.2 and 3.3), indicating that the change of glycosylation pattern seems to be associated with the cellular transition and differentiation of gonocytes within a week after birth in mice. Since DBA–

positive cells were decreased and UCHL1 and DDX-positive germ cells were dramatically increased at 3 dpp to 5 dpp (Fig. 3.4), gonocytes, which are mitotically quiescent state after birth, resume the mitotic division at this stage. Although germ cell potentials of DBA-negative cells by transplantation assay to immune-deficient mouse testis was not examined in this experiment, the surface glycan epitopes can be useful to define cell characteristics during differentiating processes from gonocytes to spermatogonia.

The change of DBA-binding pattern in a part of gonocyte population detected at 2 dpp from diffused pattern to the condensed pattern in gonocytes cytoplasm (Fig. 3.3), and the number of gonocytes with the condensed DBA signals gradually increased at 5-7 dpp (Fig. 3.5). Because the condensed form of glycan epitopes in the Golgi apparatus observes after the onset of spermatogenesis in cattle (Ertl & Wrobel 1992), the intense signals of DBA-binding in the cytoplasm of gonocytes might be caused by the accumulation of glycan epitopes in the Golgi complex. The distributional changes of these glycan epitopes could be a common feature at the initial stage of spermatogenesis in mammalian species. These results suggest that the change of glycosylation in gonocytes at 2 dpp is associated with the appearance of spermatogonia. This observation is concomitant with the timing of c-Kit expression in gonocytes at 2 dpp (Yoshida et al. 2006). The difference of DBA-binding affinity to differentiating gonocytes may be a common feature in mammals as pig (Goel et al. 2008) and cattle (Kim et al. 2013). These molecular features of glycan epitopes reflects the characteristic changes of gonocytes through the cell surface interaction, which is associated with cell survival and proliferative activity during the initial stage of early spermatogenesis (Kim et al. 2013). Ohmura et al. (2004) revealed that mouse gonocytes represent heterogeneity in the distinct cell population with the expression pattern of POU5F1. These different characteristics of gonocytes at 0.5 dpp affect a functional difference of gonocytes,

and are associated with germ cells potential undergoing spermatogenesis after transplantation into recipient testis (Ohmura et al. 2008).

The first step in the differentiation process of gonocytes has been focused in the neonatal mouse testis. It have been generally accepted that a population of gonocytes gives rise to either undifferentiated type A spermatogonia (As, Apr and Aal spermatogonia) or the differentiating type A spermatogonia (type A1 and/or A2 spermatogonia) (de Rooij 1998, Yoshida et al. 2006). In this study, the DBA-positive cells with the condensed signals at 2 dpp were progressively lost UCHL1 expression and located in the central part of the seminiferous tubules at 5 dpp to 7 dpp (Fig. 3.5), while DDX4 expression was continuously detected in the DBA-positive cells (Fig. 3.6). UCHL1 expression is restricted on gonocytes and type A spermatogonia in mouse testis (Kon et al. 1999) and the DDX4 expression can observe in gonocytes, type A spermatogonia and spermatocytes in developing mouse testis (Toyooka et al. 2000). These evidences indicate that the condensed form of DBA signals can be observe on the earlier step of gonocytes differentiation and DBA-positive cells differentiate progressively into differentiating spermatogonia. Although the precise role of the glycosylation on gonocytes remained to be elucidated in this experiment, it is suggest that the differential distribution of glycan epitopes may involve in cell differentiation and the appearance of the distinct cell lineages of gonocytes at the onset of spermatogenesis.

Stimulated by retinoic acid gene 8 (*STRA8*) is induced by retinoic acid (RA) and has been detected on embryonic germ cells before onset of meiosis (Bowles et al., 2006; Koubova et al., 2006). Postnatally, the expression of STRA8 protein is detected as a spermatogonial marker, and has a crucial role in inducing meiosis in both spermatogenesis and oogenesis (Oulad-Abdelghani et al. 1996, Anderson et al. 2008). Interestingly, in this study, the expression of STRA8 protein at 7 dpp was detected in DBA-positive cells, which

had the condensed form of DBA signal on the cytoplasm of gonocytes (Fig 3.6A and B). Indeed, the transition of gonocytes into differentiating type A spermatogonia has been observed at first week after birth (Yoshida et al., 2006) and may be associated with STRA8 expression both *in vitro* (Zhou et al., 2008a) and *in vivo (Zhou et al., 2008b)*. In addition, the DBA signals in the adult testis were detected as condensed dots on spermatocytes, which are accompanied by the chromatin condensing of chromosome (Fig. 3.6C). In fact, the transition of gonocytes into differentiating type A spermatogonia is observed at the first week after birth (Yoshida et al. 2006) and is associated with STRA8 expression both in vitro (Zhou et al. 2008a) and in vivo (Zhou et al. 2008b). The condensation of the glycan epitopes on the cytoplasm of gonocytes reflects the characteristic changes corresponding to the initial process of the meiosis derived from gonocytes. Therefore, molecular features of glycan epitopes the initial differentiation of gonocytes is coincident with the dynamical change of glycan epitopes may be a sign for the alteration of characteristics. These molecular features indicate that gonocytes consist of a heterogeneous population and the modification of glycan epitopes requires for the progressive differentiation of spermatogenesis (Fig. 3.7). The phonotypic consequence for glycan distribution provides us a useful tool for analyzing the mechanisms of germ cell differentiation at the primitive meiotic stage of spermatogenesis

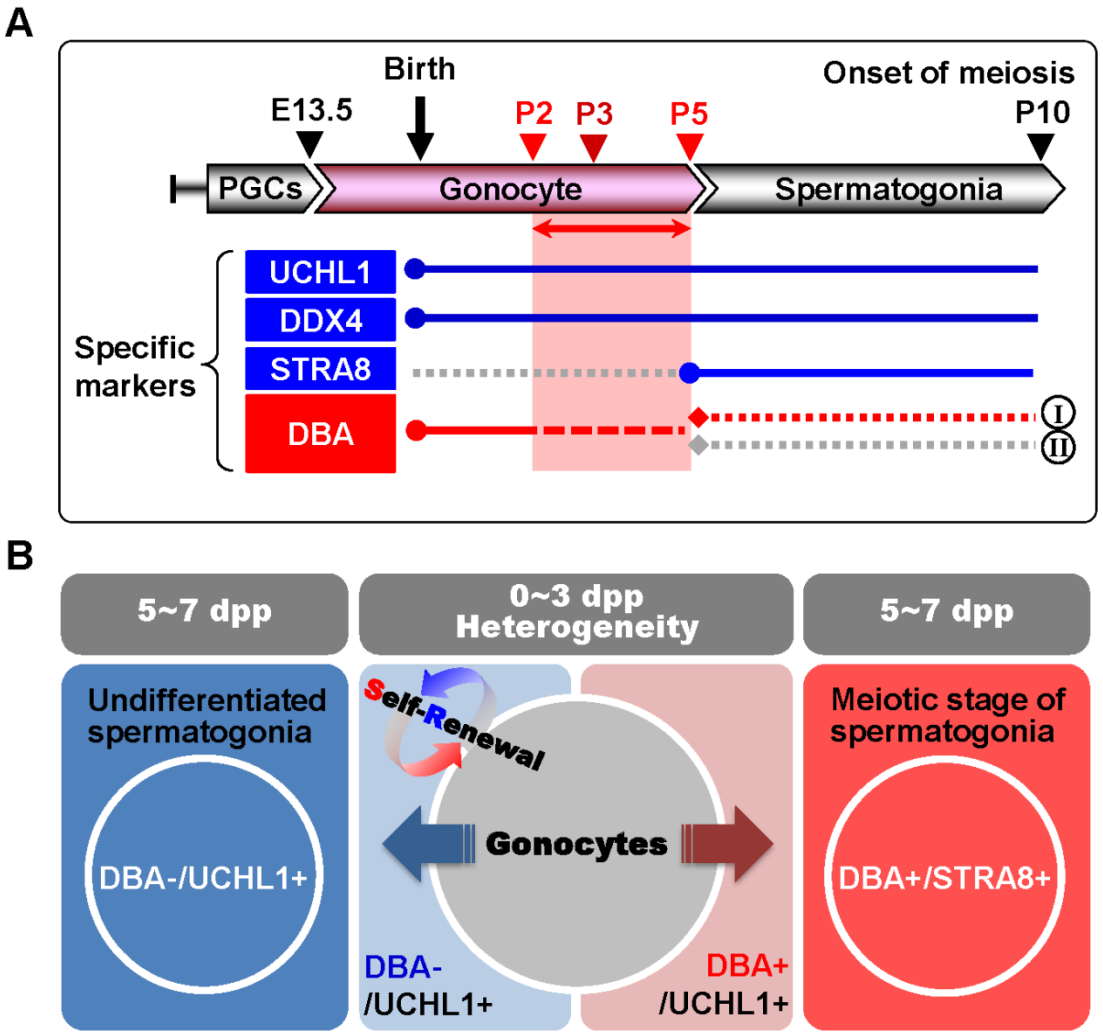

Figure 3.7 Schematic diagram of the initial process of gonocyte differentiation identified by DBA-binding affinity in neonatal mouse testis. (A) Schematic representation of DBA-binding affinity and the expression of germ cell markers, such as UCHL1, DDX4 and STRA8 during the development of germ cells. (B) A model for initial differentiation of gonocytes in the neonatal mouse testis. Gonocytes that present glycan epitopes at 0.5 to 1 dpp exhibited homogenous characteristics, which were consisted of the DBA-positive (DBA+/UCHL+) and DBA-negative cells (DBA-/UCHL+). Undifferentiated spermatogonia are derived DBA-negative cells as a source of next coming spermatogenesis, while differentiating spermatogonia are originated DBA-positive cells followed by the meiotic phase of spermatogenesis.

CHAPTER 4

Effects of extracellular matrices and lectin DBA on cell adhesion and self-renewal of bovine gonocytes cultured *in vitro*

4.1 Introduction

A population of germ cells has the unique ability to transmit genetic information to the next generation. Gonocytes are primitive germ cells that are present in the early stage of the neonatal testis and that give rise to spermatogonia. Spermatogonia have the potential for self-renewal and differentiation to spermatozoa, thereby initiating spermatogenesis. In rodents, gonocytes growing in culture acquire the characteristics of spermatogonia, exhibit stem-cell potential as indicated by their self-renewal (Kanatsu-Shinohara et al., 2003; 2005), and can contribute to spermatogenesis after transplantation into immune-deficient nude mouse testis (Orwig et al., 2002a; 2002b). Similarly, gonocytes that present in the bovine testis of 3-month-old are only germ cells, which are replaced to the spermatogonia at around 4 months of age, and initiation of spermatogenesis observed between 16 and 24 weeks (Curtis and Amann, 1981). However, in domestic animals, little is known about whether gonocytes have stem-cell activity during germ cell development. Culture conditions for maintaining germ cells have been established for various species including mouse (Kanatsu-Shinohara et al., 2005; Kubota et al., 2004; Nagano et al., 1998; 2003), rat (Hamra et al., 2005), hamster (Kanatsu-Shinohara et al., 2008a) and rabbit (Kubota et al., 2011). In domestic animal species, however, culture systems have not been available and cell lines such as embryonic germ (EG) cells in mouse have not been established.

In the testis, the dynamic events during spermatogenesis occur through the basement membrane of the seminiferous tubule and the interaction with Sertoli cells. In fact, the basement membrane of the seminiferous tubule is composed of extracellular matrix (ECM), whose major components are collagen and laminin (Siu and Cheng, 2004). Recent studies have revealed that adhesion molecules on the surface of SSCs specifically recognize ECM components, which have been used to identify and purify the population of germ cells in mixed testicular cells (Hamra et al., 2005; Orwig et al., 2002c; Shinohara et al., 1999). Furthermore, adhesion molecules, such as β1- and α6-integrin are known to be receptors of laminin. These molecules, which are present on the surface of mouse SSCs, support the long-term proliferation of SSCs in culture (Kanatsu-Shinohara et al., 2005; Shinohara et al., 1999) and play critical roles in the reconstruction of the stem cell niche after transplantation into immunodeficient mouse testis (Kanatsu-Shinohara et al., 2008a). Therefore, the adhesion of cells to ECM molecules seems to be associated with their survival and proliferation, both *in vitro* and *in vivo*. However, in the case of cattle, little is known about the mechanism by which germ cells adhere to ECM matrices.

One approach to distinguishing and characterizing germ cells in a mixed testicular cell population is to identify a stage-specific glycosylation event. A lectin, DBA, which recognizes a terminal GalNAc residue (Piller et al., 1990), is a specific marker for germ cells such as gonocytes and type A spermatogonia in both pig (Goel et al., 2007) and cattle (Ertl and Wrobel, 1992; Izadyar et al., 2002). In addition, DBA can be used to enrich germ cells by using magnetic-activated cell sorting (MACS) (Herrid et al., 2009). Therefore, germ cells isolated by DBA can be a useful model for understanding the roles of cell surface glycans in adhesion and proliferation of germ cells both *in vivo* and *in vitro*.

In domestic animals, a procedure for a long term culture of germ cells has not been

established. To achieve this, the expressions of vital pluripotency-related genes such as *NANOG* and *POU5F1* are essential, but their expressions gradually decrease as the passage number increases (Goel et al., 2009). The pluripotent state in cultured germ cells can be supported by using ECM components that interact with adhesion molecules on the cell surface (Chai and Leong, 2007), which suggest that some cell surface molecules can regulate the expression of genes associated with a pluripotent state in cultured germ cells. However, the effects of biomaterials, such as ECM molecules and DBA, on the adhesion, proliferation and stem cell potential of germ cells remain unknown in domestic animals.

In the present study, we tested the hypothesis that adhesion molecules including carbohydrate chains on the surface of germ cells affect cell survival and proliferation in culture. Our results suggest that the terminal glycan residues of cell surface carbohydrates are involved in the proliferation and the stem cell potential of bovine gonocytes in culture.

4.2 Materials & Methods

4.2.1 Collection of the testes and the isolation of gonocytes

Testes were collected from Holstein bulls (Bos taurus) aged 3 months old from a local farms and were immediately placed in DMEM/F12 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 100 IU/ml^{-1} penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 mg/ml^{-1} streptomycin (Sigma-Aldrich), 40 mg/ml^{-1} gentamycin sulfate (Sigma-Aldrich) and 15 mM HEPES (Wako, Osaka, Japan). The collected testes were transported to the laboratory at 4℃ within 24 h. The part of the testis was fixed with Bouin's fixative or 4% (w/v) paraformaldehyde (PFA) solution for immunohistochemical analysis.

To collect testicular cells, the testes were treated with three-step enzymatic digestions

and isolated cells were subjected to the discontinuous density gradient Percoll centrifugation as described previously with some modifications (Fujihara et al. 2011). Briefly, to obtain a testicular cell suspension, the decapsulated testicular tissue was minced into small pieces and treated with a first enzymatic solution that was supplemented with 2mg/ml^{-1} collagenase (type IV; Sigma-Aldrich) and 1 mg/ml^{-1} deoxyribonuclease I (DNase I; Sigma-Aldrich) in DMEM/F12 for 30 min. at 37℃. Testicular cells were washed 3 times in DMEM/F12 and sequentially digested with a second enzymatic solution containing 2mg/ml^{-1} collagenase (type IV; Sigma-Aldrich), 2 mg/ml^{-1} hyaluronidase (Sigma-Aldrich) and 1 mg/ml^{-1} deoxyribonuclease I for 30 min at 37℃ and washed with DMEM/F12. The collected cells were incubated with third enzymatic solution (0.25% trypsin and 0.53 mM EDTA in PBS) containing 5 mg/ml^{-1} deoxyribonuclease I for 10 min at 37℃, washed with DMEM/F12, filtered with 50 ㎛ nylon meshes (Kyoshin Rikoh, Tokyo, Japan), and the isolated cells were subjected to the discontinuous density gradient Percoll centrifugation. Gonocytes were fractionated between 40 to 50% and identified by DBA-staining and morphological definition with large diameter in cell size. Gonocytes were collected 1 × 10^6 cells per one gram of the testis tissue and were enriched ≤40% by the density gradient purification. The viability of purified cells was ≥95%, as determined by trypan blue exclusion assay.

4.2.2 In vitro culture of gonocytes

Freshly collected gonocytes were seeded at a density of 2 × 105 cells/cm^{-2} onto culture dishes (Iwaki, Tokyo, Japan). The culture medium used was DMEM/F12 supplemented with 10 ㎍/mL^{-1} insulin (Sigma-Aldrich), 10 ㎍/mL^{-1} apotransferrin (Sigma-Aldrich), 100

IU/mL^{-1} penicillin (Sigma-Aldrich), 50 μg/mL^{-1} streptomycin (Sigma-Aldrich), 40 μg/mL^{-1}, gentamycin sulfate (Sigma-Aldrich), single strength non-essential amino acid solution (Gibco, Invitrogen), 1mM pyruvate (Sigma-Aldrich), 1.5 $\mu\ell$/ml^{-1} 60% (w/v) sodium lactate (Sigma-Aldrich), 0.01mM β-mercaptoethanol (Wako), 20 ng/mL^{-1} basic fibroblast growth factor (bFGF; Upstate, Temecula, CA, USA), 20 ng/mL^{-1} glial-derived neurotrophic factor (GDNF; R&D System, Minneapolis, MN, USA), 50 ng/mL^{-1} epidermal growth factor (EGF), 1% (v/v) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) and 15% (v/v) knockout serum replacement (KSR). The culture medium was changed every other day and passaged at every 7 to 10 days interval using 0.25% (w/v) trypsin and 0.53 mM EDTA solution or mechanical dissociation methods using a fire-polished Pasteur pipette. Cells were cultured in a CO2 incubator at 37℃ in a water-saturated atmosphere with 95% air and 5% CO2.

4.2.3 Preparation of ECM matrix plates and assessment of binding affinity of germ cells

Culture dishes were pre-coated with ECM molecules (0.2% (w/v) gelatin (GN) (Sigma-Aldrich), 20 μg/mL^{-1} laminin (LN) (Sigma-Aldrich) and 10 μg/mL^{-1} poly-L-lysine (PLL) (Sigma-Aldrich) and 30 μg/mL^{-1} DBA (Vector Laboratories, Burlingame, CA, USA) for overnight at 37℃, then washed with PBS and were blocked with 5% BSA in PBS for 1 h at 37℃ to prevent non-specific binding.

To analyze the binding affinity of gonocytes to culture dishes, freshly collected gonocytes were plated in 4-well or 24-well culture dishes (Iwaki) pre-coated with different ECM molecules. The experimental process to access the binding affinity of gonocytes has represented on Fig. 4.1. In briefly, cells were incubated for 4 h at 37 ℃ in an adherent medium, which was DMEM/F12 supplemented 10% FBS without KSR and growth factors to

enhance the attachment of germ cells on ECM matrices. After 4 h of culture, floating cells were discarded, and adhered cells were gently washed and collected in the culture medium. Adhered cells were characterized by immune-cytochemical staining to distinguish germ-cell and somatic-cell populations. Antibodies were used for germ-cell markers (UCHL1 and DBA) and Sertoli-cell marker (VIMENTIN). The average numbers of positive cells for germ cell-specific markers were counted in the microscopic field (magnification: 200x), which were randomly selected six fields per sample. Each experiment was done using single animal and the experiment was repeated at least 4 times using 4 to 5 animals.

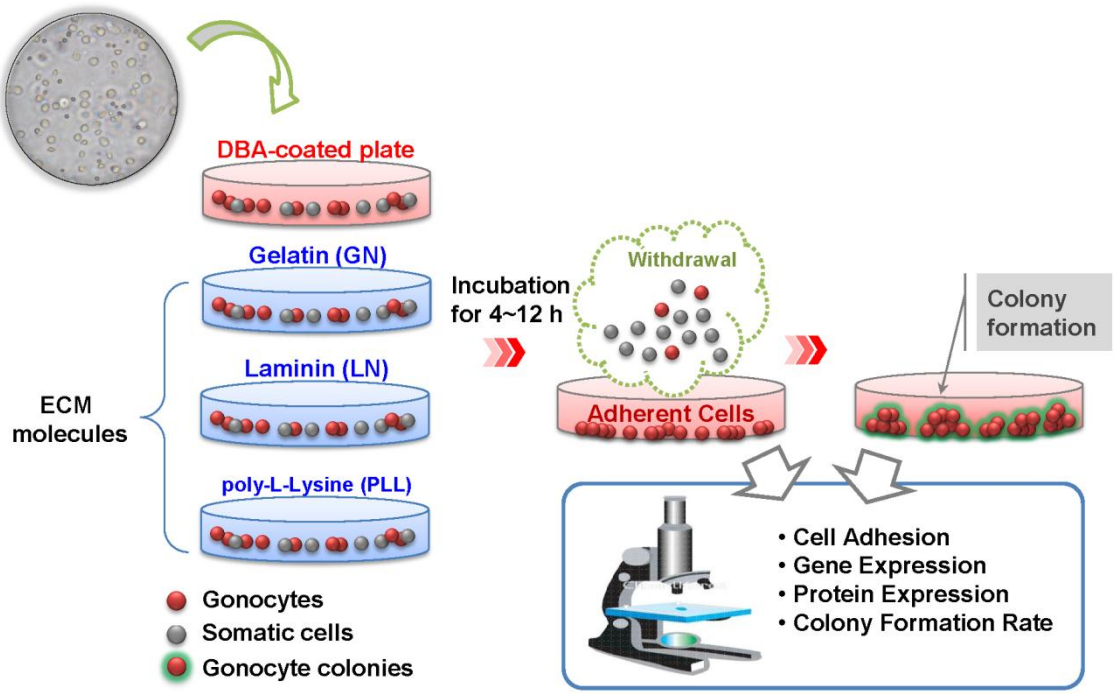

Figure 4.1 A schematic representation for experimental procedure in this study. Freshly isolated testicular cells were cultured to enrich gonocytes on the DBA and different ECM plates for 4 to 12 h. Supernatant was removed and estimated the number of adherent cells on the plates. Adherent of testicular cells was then cultured and subjected other experiment to analyze the efficiency of colonies formation and pluripotency of germ cells.

4.2.4 Assessment of colony formation on the different ECM matrices

Freshly isolated gonocytes were seeded at a density of 2×10^5 cells/cm^{-2} onto 4-well, 24-well or 35 mm dishes. Gonocytes were incubated in the adherent medium on the pre-coated dishes, which were pre-coated with different ECM matrices and DBA for 12 h at 37℃. Gonocytes were then pre-incubated with DBA (30μg/mL^{-1}) for 30 min at 37 ℃ to neutralize GalNAc residues on the surface of gonocytes. After pre-incubation, gonocytes were seeded at a density of 2×10^5 cells/cm^2 onto 4-well, 24-well or 35 mm dishes. DBA pre-treatment cells were incubated on the GN plates (D30_GN) and DBA plates (D30_DBA) for 12 h at 37℃. After 12 h of culture, floating cells were decanted and the adhered cells were washed with culture medium, and then cultured with the adherent medium for another 4 to 7 days on different ECM matrices or DBA. To examine the glycan epitopes on colony formation, gonocytes were then pre-incubated with DBA (30μg/mL^{-1}) for 30 min at 37 ℃ to neutralize GalNAc residues on the surface of gonocytes. After pre-incubation, gonocytes were seeded at a density of 2×10^5 cells/cm^2 onto 4-well, 24-well or 35 mm dishes and were incubated on the GN plates (D30_GN) or DBA plates (D30_DBA) for 12 h at 37℃. The culture medium was changed every 2 days. At 5 days, the total numbers of colonies were counted on each well of a 4-well or 24-well plate to obtain the average number of colonies. The above procedure was replicated 4 times for the each group.

4.2.5 Immunochemistry of testicular tissues and cultured gonocytes

Gonocytes were identified in the testicular tissues and in cultured testicular cells using DBA-FITC (1:50; Vector Laboratories, Burlingame, CA, USA) and anti-UCHL1 (PGP9.5; 1:100; Biomol, Exeter, UK). The presence of Sertoli cells in cultured testicular cells were

confirmed by using anti-VIMENTIN (clon v9, 1:100; Sigma-Aldrich). The expression of pluripotency specific-markers on gonocytes in bovine testis and cultured testicular cells was examined using anti-NANOG (1: 200; Chemicon International, USA) and anti-POU5F1 (1:50; C-10, Santa Cruz Biotechnology, CA, USA), as described previously (Goel et al. 2008; Fujihara et al. 2011). Briefly, testis sections were fixed with Bouin's fixative or 4% PFA, washed several times with 0.2% (v/v) Tween 20 in TBS (TBS-T), incubated in 5% (w/v) BSA in TBS for 90 min to block non-specific binding, incubated with the DBA-FITC and primary antibodies overnight at 4˚C, washed with TBS-T three times, incubated with the corresponding secondary antibody as an anti-rabbit IgG antibody conjugated with Alexa 546 (1:500; Molecular Probes, Eugene, Oregon, USA) and anti-mouse IgG antibody conjugated with Alexa 546 (1:500; Molecular Probes, Eugene, Oregon, USA) for 1 h at 37℃, rinsed three times with TBS-T, stained with Hoechst 33342 (Sigma-Aldrich) for 10 min, mounted with 50 % glycerol in PBS and observed under an immune-fluorescence microscope (Olympus BX 50, Tokyo, Japan).

Cultured cells were examined for the presence of gonocytes by germ-cell-specific markers (DBA, and anti-DDX4) and for stem-cell potential by pluripotent-specific markers (anti-NANOG and anti-POU5F1). Samples were fixed with Bouin's fixative or 4% PFA, washed several times with TBS-T, incubated 0.3% (v/v) H_2O_2 in PBS for 15 min to block endogenous peroxidase activity, washed with PBS several times, incubated in 5% (w/v) BSA in PBS for 30 min to block non-specific binding and incubated with DBA and primary antibodies overnight at 4℃. The primary antibodies were anti-NANOG (1:200 dilution), anti-POU5F1 (1:50 dilution), and anti-DDX4 (1:300, Chemicon, USA). After incubation with primary antibodies, samples were washed with TBS-T three times, incubated with substrate-chromogen mix for DBA or the corresponding HRP-conjugated secondary antibodies, i.e.,

sheep anti-rabbit IgG (1:100; GE Healthcare, Buckinghamshire, UK), sheep anti-mouse IgG (1: 100; Amersham Biosciences, UK) for 1 h at room temperature, rinsed several times with TBS-T, mixed with substrate-chromogen for 3-5 min to colorimetrically measure peroxidase activity, washed with TBS several times, counterstained with hematoxylin, mounted on slides, and observed under the microscope (Olympus BX 50, Tokyo, Japan).

To examine a stem-cell-potential of gonocytes in cattle, purified gonocytes were double stained with DBA-FITC, anti-UCHL1, anti-NANOG and anti-POU5F1 antibodies using immune-fluorescence labeling as described above.

4.2.6 RT-PCR analysis

Testicular cells were cultured for 4 days on the different ECM matrices. Total RNAs were prepared from these cells using a ToTally RNA kit (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. RNAs were also isolated from 3-month-old testes as a positive control (T). Oligo (dT) primers and RNase OUT (both from Invitrogen) were added to the RNA solution, incubated for 5 min at 65℃ and set on ice. For reverse transcription, ReverTra Ace (MMLV reverse transcriptase RNaseH-; Toyobo) was added to the RNA solution and incubated for 10 min at 30℃, for 60 min at 42℃, and for 5 min at 99℃ (RT+). At the same time, the reaction without the addition of ReverTra Ace was done to check genomic DNA contamination (RT-). The PCR amplification was carried out on 2 μl of cDNA per 20 μl of PCR reaction mixture containing, 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 × PCR buffer, 10 pmol of each primers and 1U of Taq DNA polymerase (ExTaq, TaKaRa, Ohtsu, Japan). The primer sequences used for the amplification of specific genes are shown in Table 1. PCR products were separated and visualized on 2 % (w/v) agarose gels containing 0.5 $\mu g/ml^{-1}$ ethidium bromide. All PCR products were sequenced to confirm their identity.

Table 1. RT-PCR primer sequences used in this study.

Gene		Primer Sequence (5´-3´)	Product Size (bp)	GenBank Accession no.
POU5F1	F	GGTTCTCTTTGGAAAGGTGTTC	314	NM_174580.2
	R	ACACTCGGACCACGTCTTTC		
NANOG	F	GACACCCTCGACACGGACACT	153	NM_001025344.1
	R	CTTGACCGGGACCGTCTCTT		
SOX2	F	GTTTGCAAAAGGGGGAAAGT	200	NM_001105463.1
	R	GAGGCAAACTGGAATCAGGA		
REX1	F	GCAGAATGTGGGAAAGCCT	209	XM_003584155.1
	R	GACTGAATAAACTTCTTGC		
UCHL1	F	ACCCCGAGATGCTGAACAAAG	236	NM_001046172.1
	R	CCCAATGGTCTGCTTCATGAA		
C-MYC	F	AGAGGGCTAAGTTGGACAGTG	346	NM_001046074.2
	R	CAAGAGTTCCGTATCTGTTCAAG		
BACT	F	TCCCTGGAGAAGAGCTACGA	364	NM_173979.3
	R	ACATCTGCTGGAAGGTGGAC		

4.2.7 Statistical analysis

All data are presented as the mean ± SEM (n= 4-5) in each group. To determine the differences among experimental groups, one-way or two-way ANOVA was performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego CA, USA). All data were subjected to Tukey's multiple-comparison test to determine the significance of differences between groups. Differences were considered to be significant at $P < 0.05$.

4.3 Results

4.3.1 Identification of gonocytes by DBA binding affinity and the UCHL1 expression in developing germ cells

To examine the DBA binding affinity and the expression pattern of pluripotent-specific markers in bovine testis, 3-month-old testes sections were immunohistochemically stained. The binding of DBA and expression of UCHL1 were observed in populations of gonocytes. These cells were easily distinguished from other somatic cell populations by two morphological features with a large nucleus and a basal location in the seminiferous tubules (Fig. 4.2A-D). DBA was found on cell surface or cytoplasmic part of gonocytes (Fig. 4.2A and B), while UCHL1 expression was observed in the germ cells (Fig. 4.2C and D).

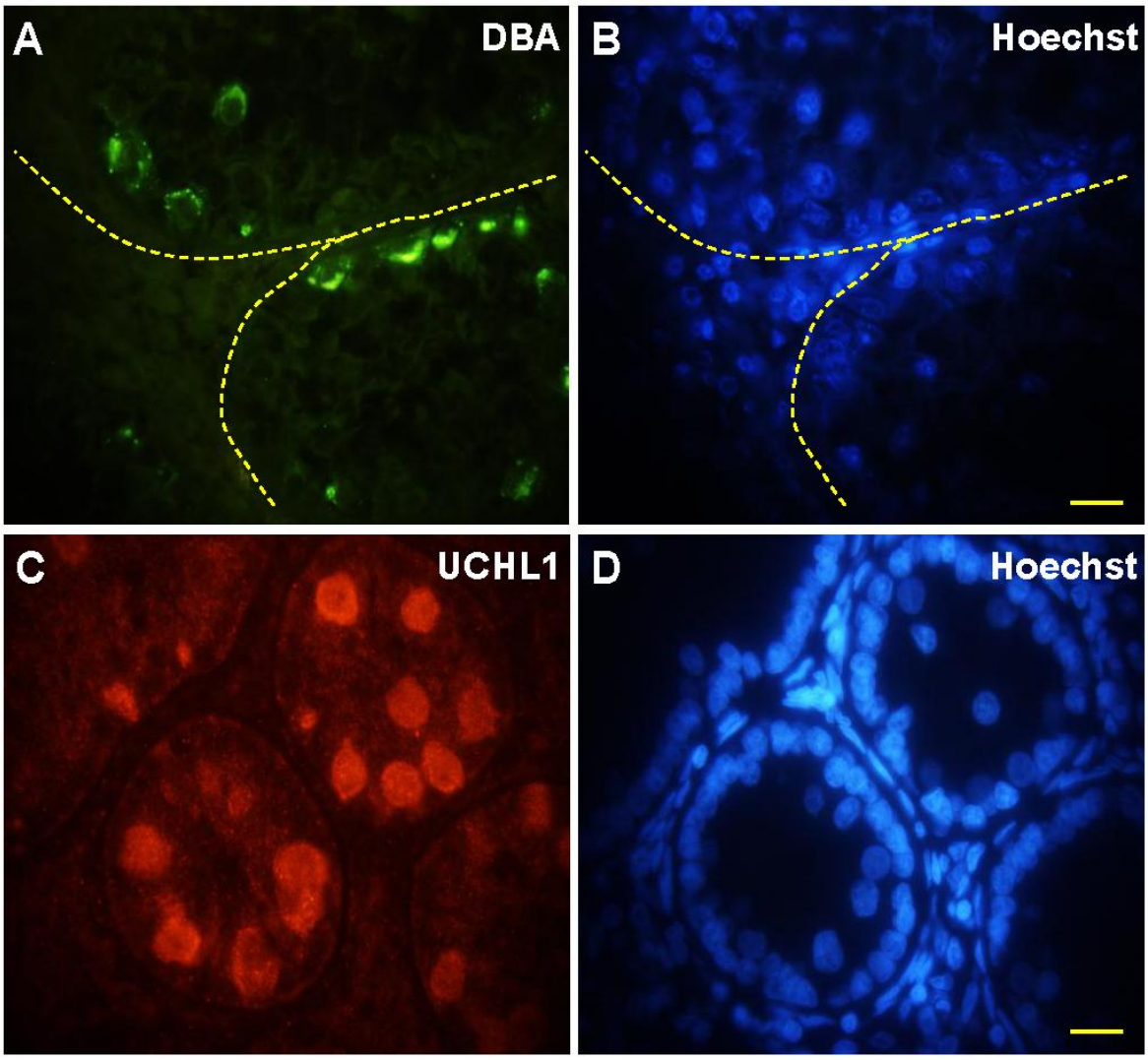

Figure 4.2 Characterization of germ cells in the bovine testis. (A-D) Germ cells in the testis stained with germ-cell markers (DBA and UCHL1). Dashed lines show the basement of the seminiferous tubules in the testis sections. (A) Some of the DBA signals were observed on the gonocytes. (B) The same sample stained with Hoechst 33342. (C) UCHL1 expression was strongly detected in the cytoplasm and nucleus of gonocytes. (D) The same sample stained with Hoechst 33342. Bar = 20㎛.

4.3.2 Characterization of stem cell potential of developing germ cells

Double-immunostaining for UCHL1 (a germ cell-specific marker) and DBA show that UCHL1 is expressed in most of the DBA-positive cells (Fig. 4.3A-C), while a small number

of UCHL1-positive cells (one is indicated by *white arrow* in Fig. 4.3A) were negative for DBA (Fig. 4.3B and C). To examine the stem-cell potential of DBA-positive germ cells, sections were double stained with DBA and anti-POU5F1 (Fig. 4.3D-F) or anti-NANOG (Fig. 4.3G-I). Most of the cells expressing POU5F1 (Fig. 4.3D) were DBA-positive (Fig. 4.3E). An example of a cell expressing POUF1 that is DBA-negative is shown by the white arrow (Fig. 4.3D-F). The POU5F1 expression was detected in most of the DBA-positive cells, but some of the POU5F1-positive cells were not shown the DBA signal (Fig. 4.3D-F). The expression of NANOG was also detected in the seminiferous tubules (Fig. 4.3*G*). Some of the NANOG-positive cells were DBA-positive and some were not (Fig. 4.3G and I). Some of the NANOG-negative cells were also DBA-positive (Fig. 4.3G-I), indicating that DBA and NANOG expression were not coincident in germ cells of the prepubertal bovine testis.

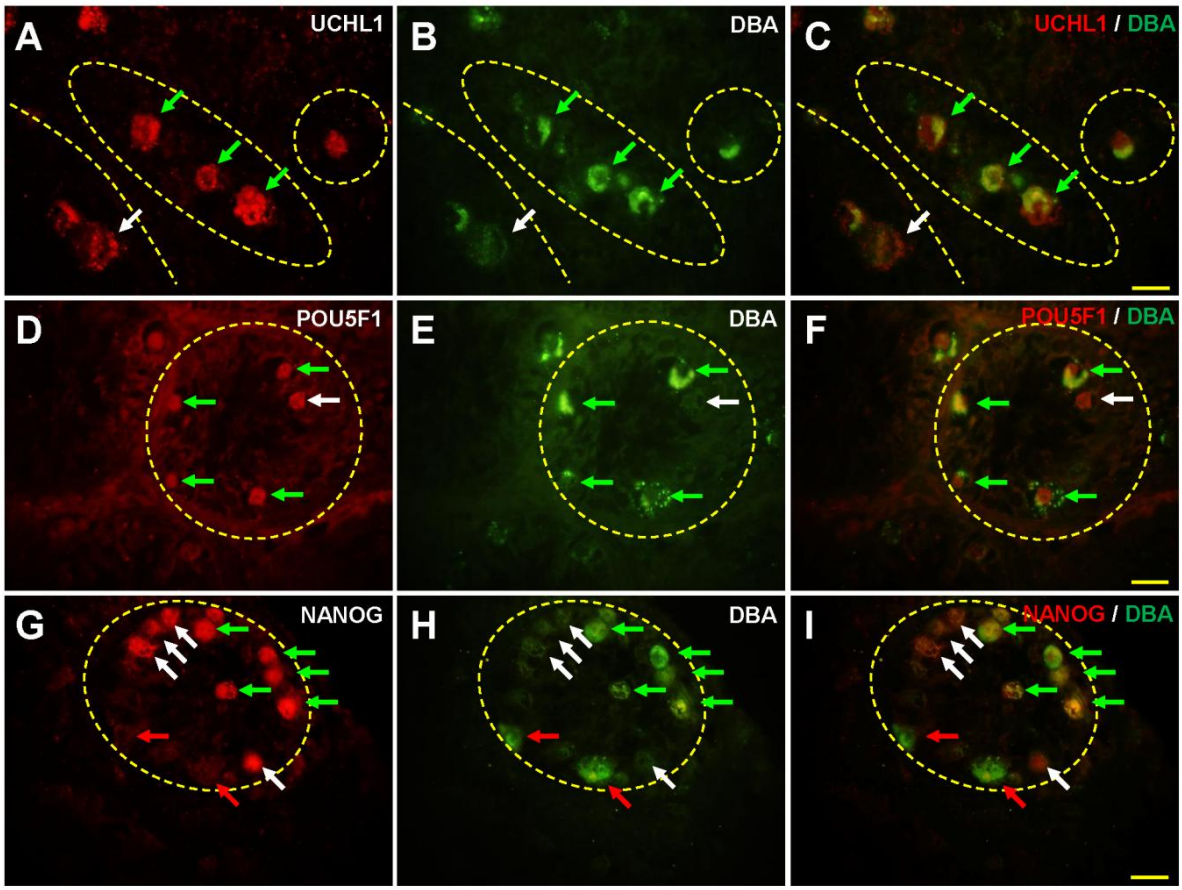

Figure 4.3 Immunolocalization of specific markers (DBA and UCHL1) for bovine germ cells in the testis. (A-C) UCHL1 expression was observed in most of the DBA-positive cells (green yellows), but was observed in only some of the DBA-negative cells (white arrows). These images were merged after double-immunostaining (C). (D-F) Double-immunostaining of DBA and POU5F1. (D-E) The expression of POU5F1 was detected on the nucleus of gonocytes in most of the DBA-positive cells (green arrows). A few POU5F1-positive cells were negative for DBA (white arrows). Merged POU5F1-staining images (F). (G-I) Double-immunostaining of DBA and NANOG. (G-H) NANOG expression was strongly detected on the nucleus of germ cells, some of which were partially positive for the DBA signal (green arrows). The expression of NANOG was observed in some DBA-negative cells (white arrows). DBA signals were detected in some NANOG-negative cells (red arrows). The image of NANOG-staining was merged with the DBA-staining image after double-immunostaining (I). Bar = 20㎛.

4.3.3 Cultivation and characterization of bovine gonocytes

Bovine gonocytes were isolated and enriched by Percoll centrifugation (Fig. 4.4A).

When the isolated cells were cultured on a GN-coated dish, they formed cell clumps at 1 day of culture (Fig. 4.4B) and formed mouse ES-like colonies by 3-4 days (Fig. 4.4C), which became compacted around 6-7 days (Fig. 4.4D) and gradually enlarged during the culture period. Most of these colonies were stained with germ cell-specific markers (DBA, Fig. 4.4E and DDX4, Fig. 4.4G) and stem cell-specific markers (POU5F1, Fig. 4.4F and NANOG, Fig. 4.4H), suggesting that gonocyte colonies in culture still have a stem cell potential. In the following passages, the colonies gradually decreased in number and disappeared by 5-7 passages.

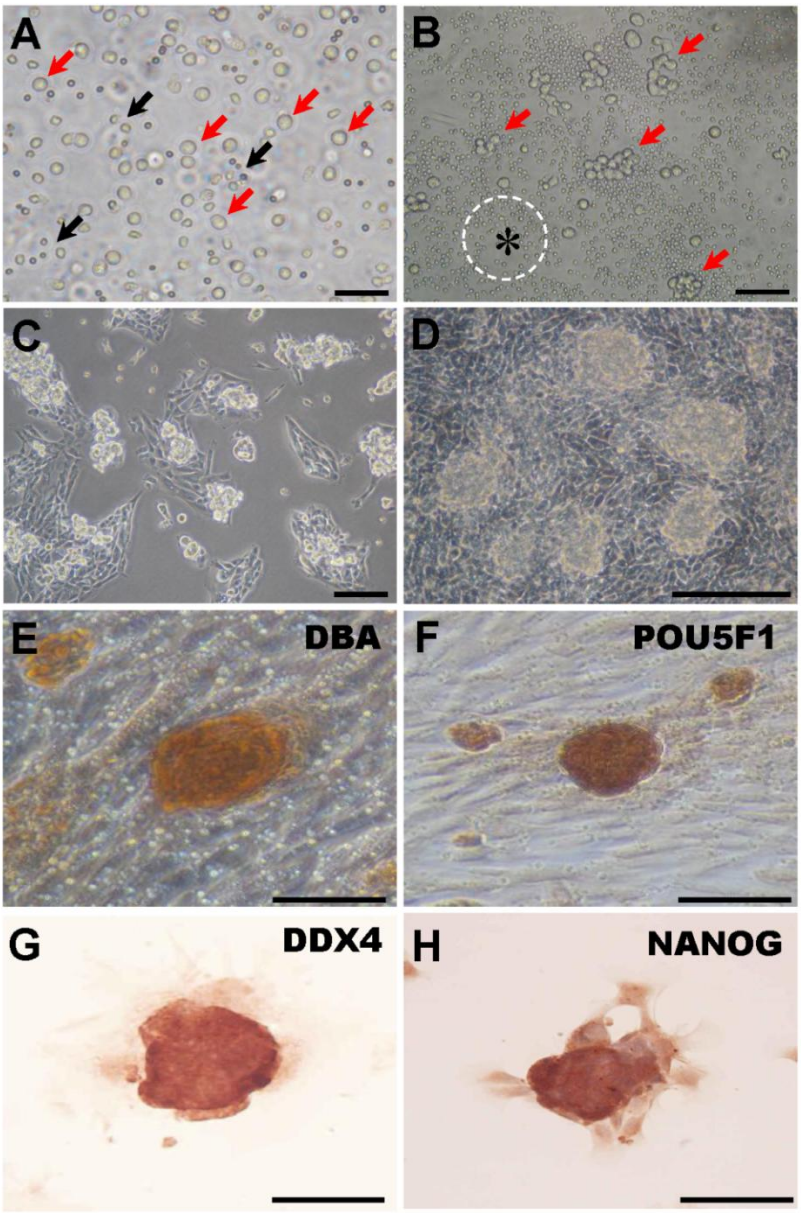

Figure 4.4 Cultivation of bovine gonocytes *in vitro*. Gonocytes from the bovine testis were collected by three-step enzymatic digestions and were cultured on gelatin-coated dishes. (A) Freshly collected testicular cells contained gonocytes (red arrows) and testicular somatic cells (black arrows). (B) Gonocytes with a larger diameter formed cell clumps (red arrows) 1 day after culture, whereas somatic cells (asterisk in the white dashed circle) did not form clumps in 1 day cultures. (C) These clumps formed mouse embryonic stem (ES) cell-like colonies at 3-4 days. (D) ES cell-like colonies of gonocytes were enlarged during 6-7 days of the culture period. (E and G) ES cell-like colonies expressed germ cell markers (DBA (E) and DDX4 (G)), pluripotency markers (POU5F1 (F) and NANOG (H)). Bar = 50㎛

4.3.4 Binding of gonocytes to DBA and different ECM matrices

The binding of gonocytes to different ECM matrices and DBA was examined at 4 h after cell plating (Fig. 4.5). The average number of testicular cells was significantly higher on the PPL plates (192.0 ±14.7 cells, $p<0.01$) and lower on the LN plates (79.0 ±9.6 cells) (Fig. 4.5A) with compared to the GN and DBA plates. The average numbers of testicular cells on the GN and DBA plates were similar (98.3 ±22.6 cells and 104.6 ±9.1 cells, respectively) (Fig. 4.5A). In the case of Sertoli cells, which are identified by staining for VIMENTIN, about equal numbers of cells bound to each of the different ECM matrices, and non-positive cells were significantly increased on the PLL plates compared to other palates (Fig. 4.5A). Although the number of attached testicular cells was highest on the PLL plates, it is interesting that the number of gonocytes was significantly higher on the DBA plates (4.21% ±0.49) than on the GN (2.03% ±0.59, $p<0.05$) and LN plates (0.75% ±0.43, $p<0.01$), but not significantly different from the number of cells on the PLL plates (2.08 ±0.52) (Fig. 4.5B). Cells that adhered to the DBA (Fig. 4.6A) and ECM plates (GN, Fig. 4.6B; LN, Fig. 4.6C; PLL, Fig. 4.6D) were detected by a germ-cell marker (DBA) and a Sertoli-cell marker (VIMENTIN) (Fig. 4.6). Gonocytes were stained only with DBA and Hoechst 33342. Cells that adhered to the DBA plate also expressed UCHL1 (Fig. 4.6E) and had a large nucleus stained with Hoechst 33342 (Fig. 4.6F). However, VIMENTIN-positive cells were not stained with DBA and had a small nucleus (Fig. 4.6E and F).

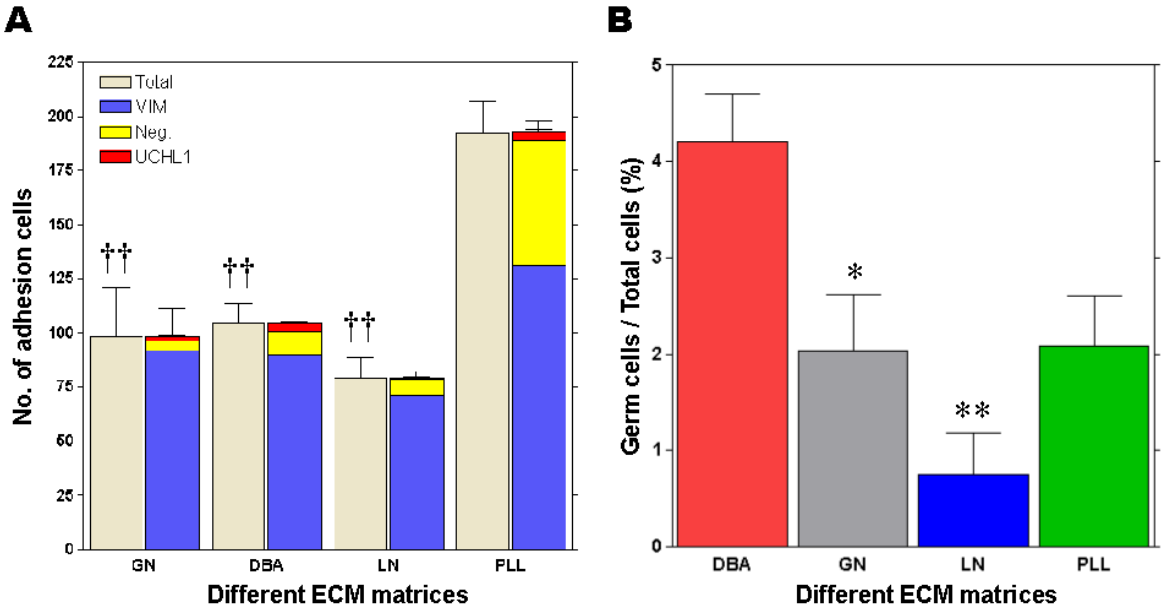

Figure 4.5 Binding affinity of gonocytes to different ECM components and DBA. Freshly collected cells were seeded on culture dishes previously coated with different ECM components and DBA, and were incubated for 4 h. Attached cells on the dishes were stained with antibodies raised against a germ-cell marker (UCHL1) and a Sertoli-cell marker (VIM: VIMENTIN). (A) Numbers of cells positive for VIM (n=4) and UCHL1 (n=4) were counted and were analyzed using graph-based visualization (mean ±SEM). Neg. (yellow bars) indicates somatic cells without staining signals. (B) Proportion of UCHL1-positive germ cells after culture on ECM- (n=4) and DBA-coated (n=5) plates (mean ±SEM).

$^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* DBA, $^{\dagger\dagger}P < 0.01$ *vs.* PLL, ANOVA and Tukey's *post-hoc* test, respectively.

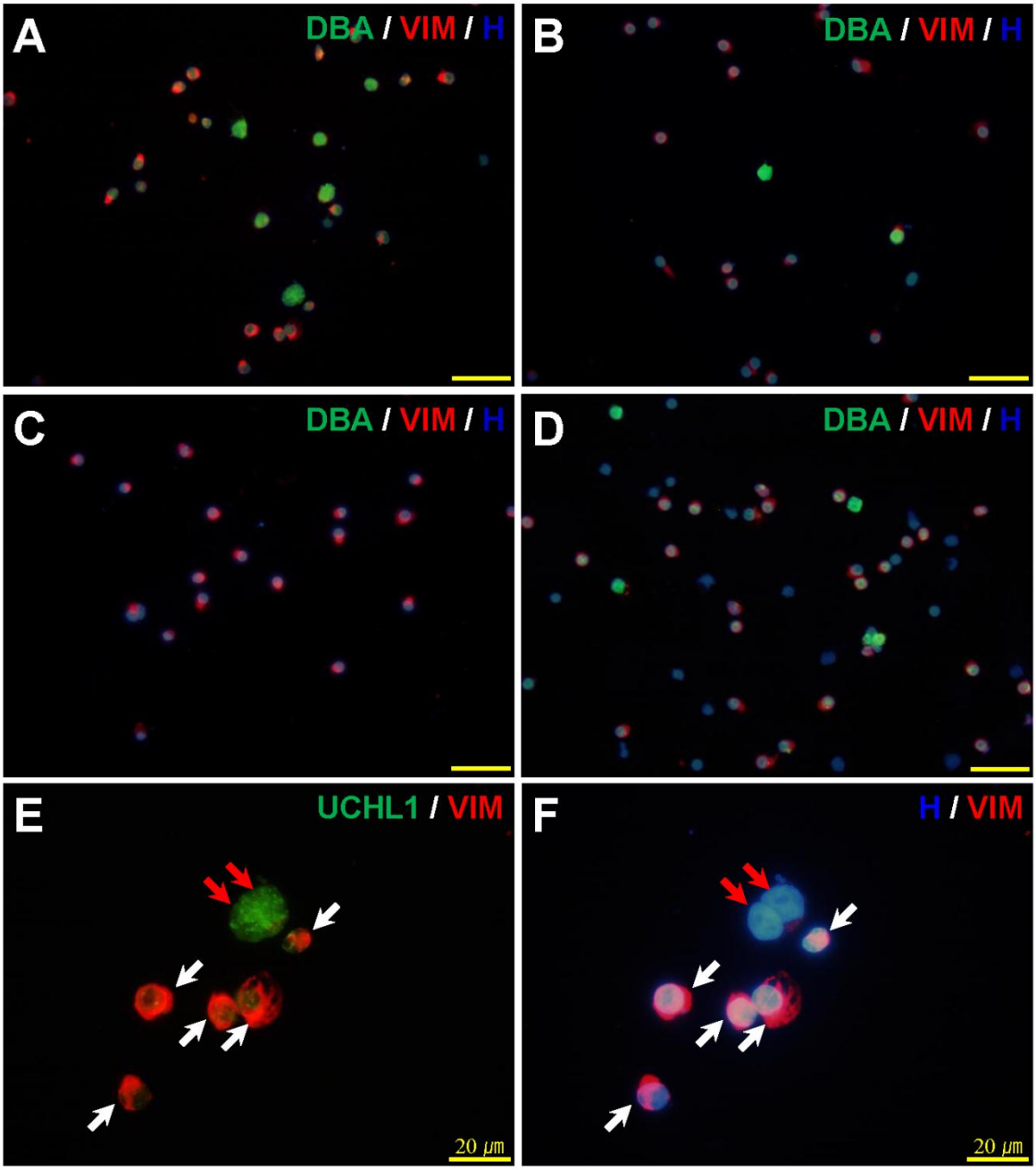

Figure 4.6 Immunocytochemical characterization of adherent cell populations on DBA and ECM plates. Cells were double stained to identify gonocytes cultured on ECM- and DBA-coated plates at 4 h after plating. Attached cells were stained with a germ-cell marker (DBA) and a Sertoli cell marker (VIM) on cover-glasses coated with DBA (A), gelatin (GN) (B), laminin (LN) (C) and poly-L-Lysine (PLL) (D). On the DBA-coated plates, germ cells were stained only with UCHL1 and overlaid with a Hoechst 33342-stained image (E and F, red arrows), and somatic cells were stained only with VIM (E and F, white arrows). H: Hoechst 33342. Bar = 50μm

4.3.5 Colony formation on the DBA and ECM plates

Freshly collected cells were cultured on plates coated with DBA and different ECMs for 5 days (Fig. 4.7A and B) and the numbers of colonies were estimated. The number of colonies on the DBA (15.8 ±1.5) and the PLL plates (14.0 ±4.4) were significantly greater than the number of colonies on the GN (6.0 ±0.4) and the LN plates (2.0 ±0.4) (Fig 4.7B). However, these colonies gradually disappeared on most plates around 7 days of culture.

After 12 h of positive selection of attached testicular cells followed by 5 days culture, colonies were observed on the DBA, GN and PLL plates, but not on the LN plates (Fig. 4.8A). Interestingly, more colonies formed on the DBA plate than on the ECM plates (Fig. 4.8A and B). The average number of colonies on the DBA plates (126.5 ±7.5) was significantly higher than the numbers of colonies on the GN, LN and PLL plates (72.5 ±0.5, $p<0.05$; 0, $p<0.001$; 33 ±13.0, $p<0.01$, respectively) (Fig. 4.8B). On the other hand, the proliferation of somatic cells was effectively suppressed on the DBA plate, but not on the ECM plates (Fig. 4.8A). When isolated gonocytes were pretreated with 30μg/ml^{-} DBA and then cultured on the DBA plates (D30_DBA) and GN plates (D30_GN), the average number of colonies were significantly decreased in both the GN (11.0 ±1.0, $p<0.001$) and DBA (30.0 ±2.0, $p<0.001$) plates (Fig. 4.8A and B). Additionally, the growth of somatic cells on the DBA was strongly suppressed on the DBA plates (Fig. 4.8A), but was not on the GN plates (Fig. 4.8A).

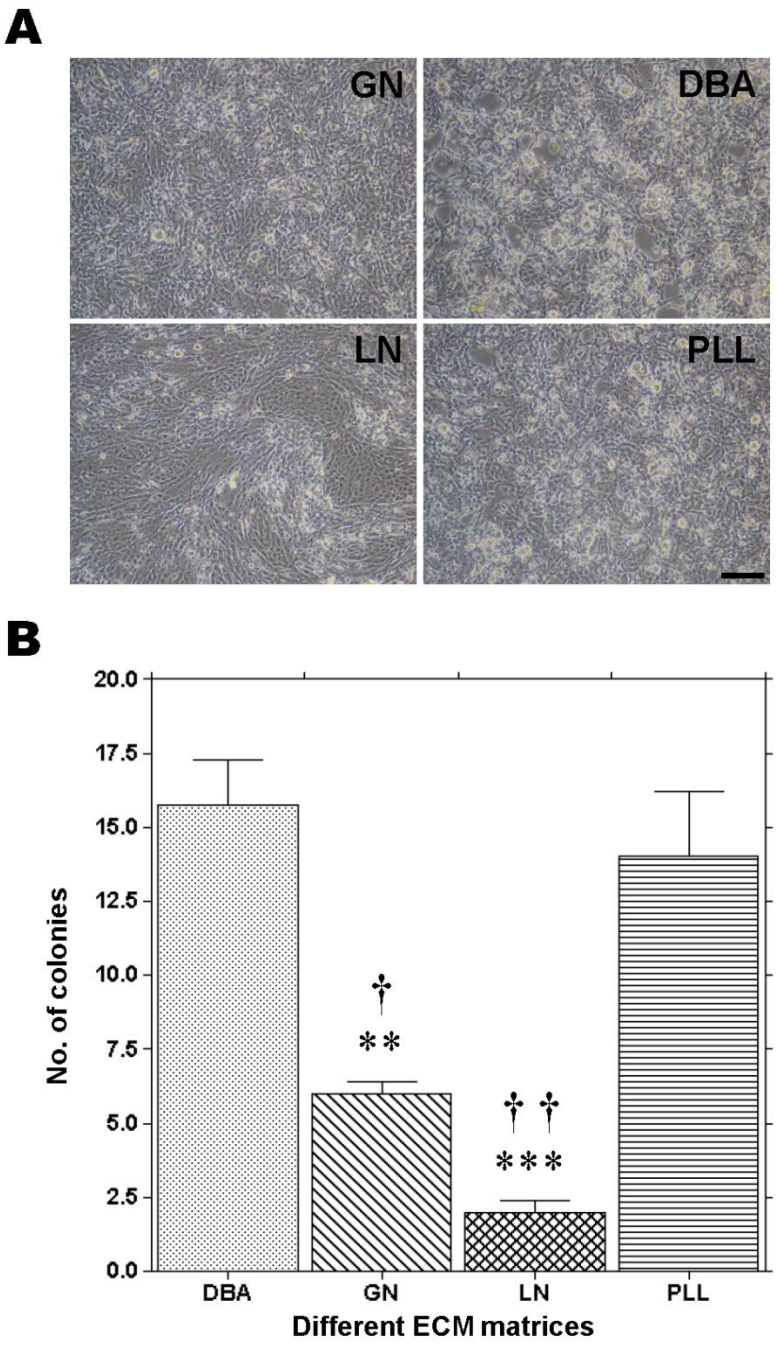

Figure 4.7 Colony formation of gonocytes cultured on different ECM and DBA plates after positive selection of germ cells. (A) Freshly collected testicular cells were cultured for 5 days on ECM (gelatin, GN; laminin, LN; and poly-L-lysine, PLL) and DBA plates. (B) Estimated numbers of colonies on ECM (GN, LN and PLL) and DBA plates (n= 4; mean ±SEM). The DBA and PLL plates had significantly more colonies than the other ECM plates (GN and LN). Bar = 50㎛.

$^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* DBA, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ *vs.* PLL, ANOVA and Tukey's *post-hoc* test, respectively.

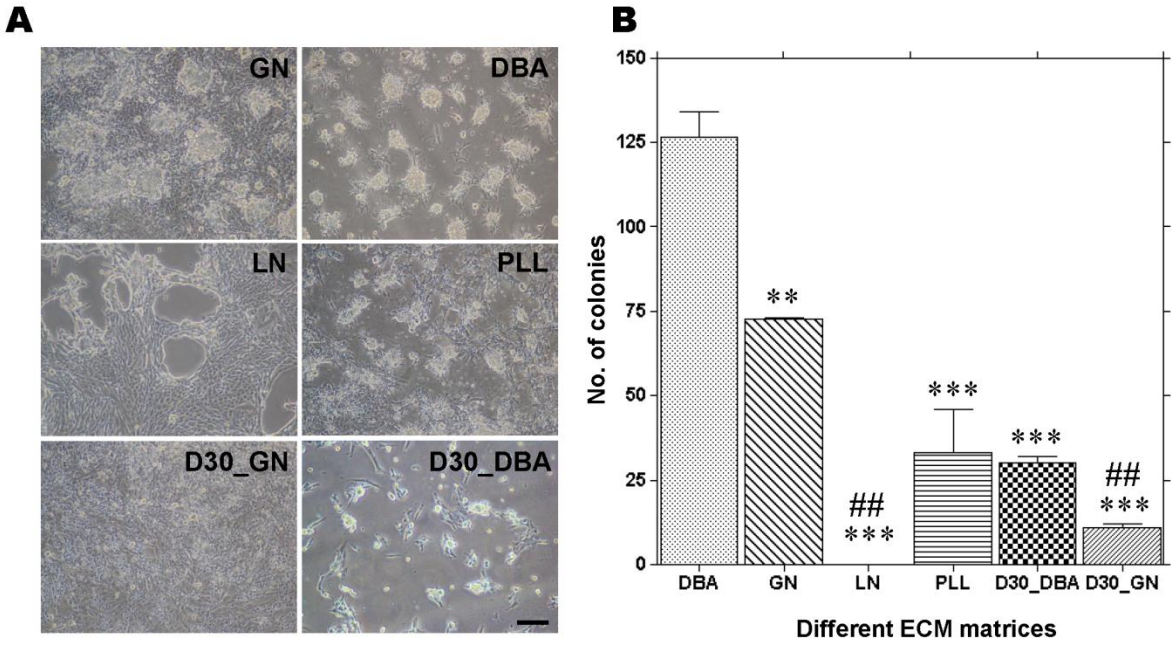

Figure 4.8 Colony formation efficiency of gonocytes on different ECM and DBA plates by the gonocyte enrichment. Freshly collected gonocytes were incubated to enrich for 12 h on the different ECM and DBA plates. After 12 h of positive selection of testicular cells, the adherent was cultured for 5 days. (A) Experimental groups were divided into two groups; one for DBA-nontreated group, in which gonocytes were simply cultured on DBA or ECM plates, and another for DBA-pretreated group, in which gonocytes were pretreated with DBA (30μg/ml^{-}) and then cultured on DBA (D30_DBA) or GN (D30_GN) plate. The growth patterns of gonocytes on the ECM and DBA plates were different. (B) Estimated numbers of colonies on the ECM and DBA plates after 5 days of culture (n= 4; mean ±SEM). The DBA plates had significantly more colonies than the GN the PLL and LN plates. Colony formation of gonocytes was significantly decreased after pre-treatment with DBA (30μg/ml^{-}) on both the GN plates (DBA30_GN) and DBA (DBA30_DBA) plates. Bar = 50μm.

**P < 0.01, ***P < 0.001 *vs.* DBA, $^{\#\#}$P < 0.01 *vs.* GN, ANOVA and Tukey's *post-hoc* test, respectively.

4.3.6 Characterization of gonocytes on DBA plates.

Three-dimensional colonies on the ECM matrices were double stained with anti-UCHL1 and anti-VIMENTIN. Most of the colonies on the DBA, GN and PLL plates were positive for UCHL1, and some of the cells in the colonies were partially positive to anti VIMENTIN (Fig. Fig. 4.9). On the LN plate, few of the colonies were UCHL1-positive, while most of the colonies were VIMENTIN-positive (Fig. 4.9).

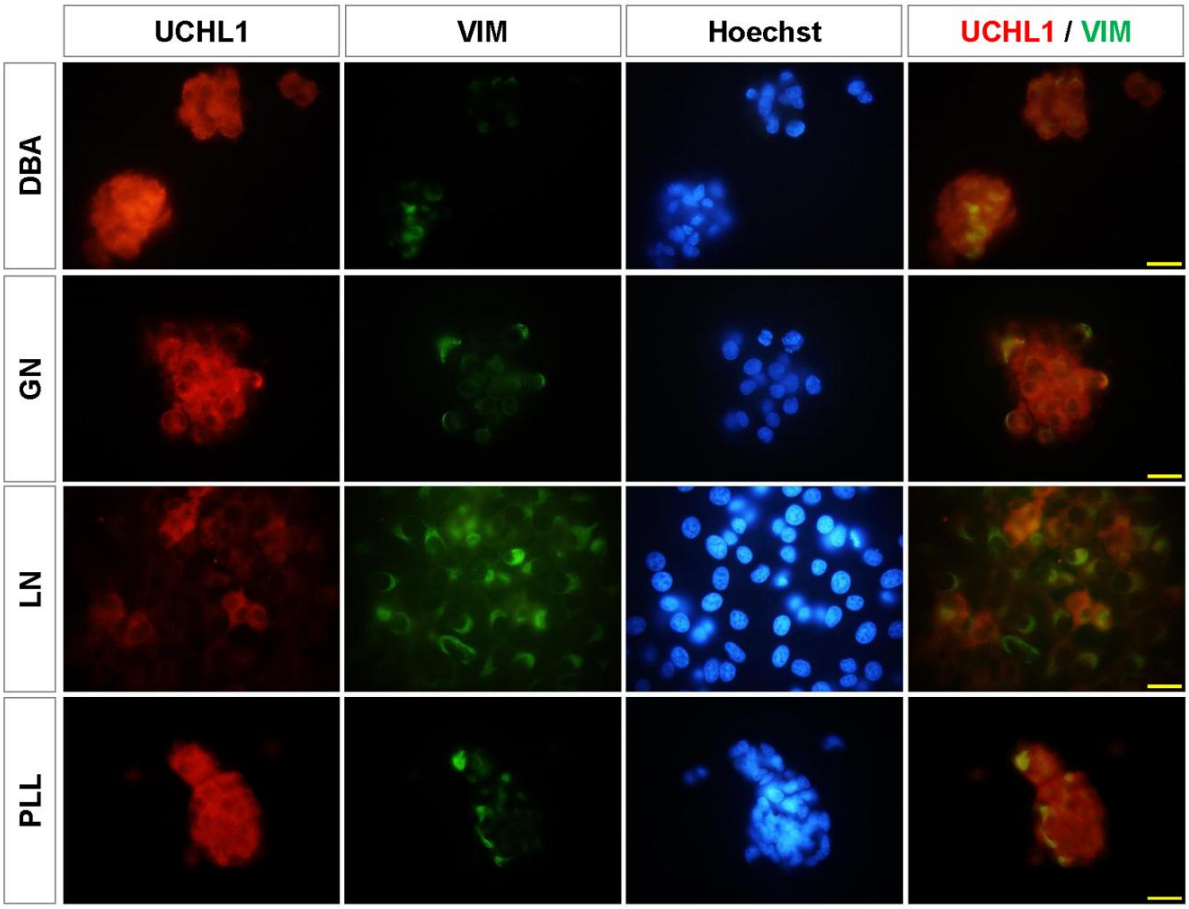

Figure 4.9 Immunocytochemical characterization of ES cell-like colonies in primary culture. Colonies that appeared on the ECM and DBA plates were stained with a germ-cell marker (UCHL1) and Sertoli cell marker (VIM: VIMENTIN). Colonies on the DBA, GN and PLL plates were positive for UCHL1 and some of the colonies expressed VIMENTIN. On the LN plate, the UCHL1 signal was weak and the VIMENTIN signal was strong. All images were merged with the image of Hoechst 33342-staining. GN: gelatin; LN: laminin; PLL: poly-l-lysine; VIM: VIMENTIN. Bar = 20㎛.

4.3.7 Pluripotent potential of gonocyte colonies on DBA plates

To estimate the stem-cell characteristics of gonocytes on the DBA plate, colonies that formed at 5 days of culture were double stained with germ-cell markers (DBA and UCHL1) and stem-cell markers (NANOG and POU5F1). Most of the colonies were strongly positive for DBA staining and were co-localized with UCHL1. These colonies that were stained with DBA also were expressed stem-cell markers NANOG and POU5F1 (Fig. 4.10), indicating that colonies of gonocytes cultured still have a stem-cell potential and germ cell ability.

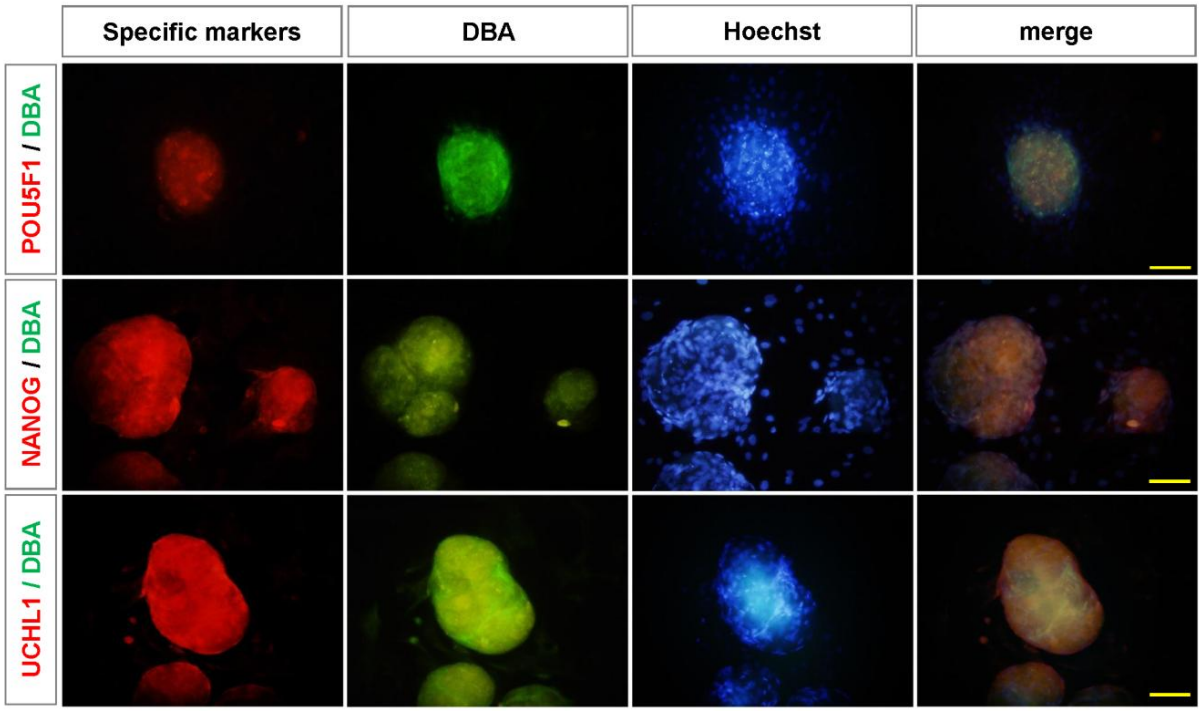

Figure 4.10 Determination of a stem-cell potential of gonocyte cultured on the DBA plates. The enriched gonocytes on the DBA plate formed colonies and gradually enlarged for culture period in primary culture. Double immunocytochemical staining was performed to identify the stem-cell potential of colonies. DBA-positive colonies were expressed UCHL1 and stem-cell markers (POU5F1 and NANOG). All images were merged with the image of Hoechst 33342-staining. Bar = 50㎛.

4.3.8 Maintenance of gonocyte colonies during long-term culture period on DBA plates

To examine the maintenance of gonocyte colonies on the DBA and GN plates, Colonies of gonocytes were passaged at every 7 or 10 days for up to 1.5 month. Colonies of gonocytes successfully formed after first passage (P1) on both the DBA (Fig. 4.11A), but were not able to maintain on the gelatin plate (Fig. 4.11C). After passage 4 (P4, up to 1.5 months), the colonies of gonocytes could be maintained on the DBA plates (Fig. 4.11B), but not on the GN plates (Fig. 4.11D). In addition, the growth of somatic cells were effectively suppressed on the DBA plate at P1 (Fig. 4.11A) and P4 (Fig. 4.11B), whereas the extensive growth of somatic cells were observed on the GN plates (Fig. 4.11D) These results show that GalNAc residues on the surface of gonocytes were associated with cell adhesion and colony formation of gonocytes on the DBA plates.

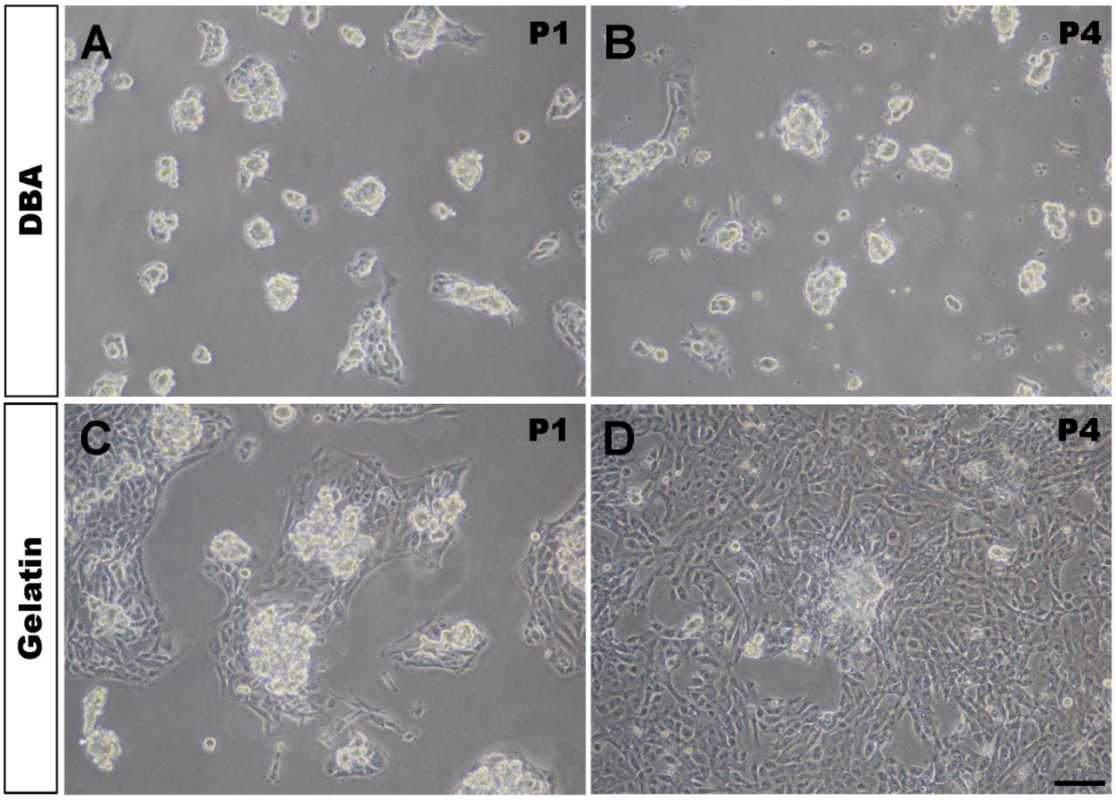

Figure 4.11 Growth pattern of cultured bovine gonocytes on DBA and gelatin plates. (A and B) Gonocytes successfully formed ES-like colonies and were maintained in both first passage (P1) (A)

and passage 4 (P4, up to 1.5 months) (B). (C and D) Colonies of gonocytes were formed with the grown of somatic cell layer on the gelatin plate at P1 (C), whereas the numbers of colonies were disappeared after P4 (D). Meanwhile, on the DBA plate, the expansion of testicular somatic cells were not observed both P1 and P4, but not on the gelatin. Bar = 50㎛.

4.3.9 RT-PCR analysis

Testes tissues and cultured cells were subjected to semi-quantitative RT-PCR analysis to identify stem cell-specific transcripts such as *NANOG, POU5F1, SOX2, C-MYC* and *REX1* (Fig. 4.12A and B). In the testis section, most of the transcripts with the exception of *C-MYC* were detected and the expression level of *NANOG* was strongly detected compared to other transcripts. Transcripts of these genes were also detected in cultured, but the expression patterns of transcripts for 4 day cultures were markedly different on the different ECM matrices and DBA. *C-MYC* transcripts were more abundant in most of the cultured cells than in freshly collected testicular cells, while *NANOG* transcripts were less abundant in the cultured cells. Among the different ECM matrices and DBA, the expression levels of *POU5F1* and *UCHL1* were markedly increased on the DBA plate, and *SOX, C-MYC* and *REX* transcripts on the DBA plate were considerably up-regulated compared to the other plates, but the expression level of *NANOG* was relatively low. On other hand, on the LN plate, the expressions of *NANOG* and *C-MYC* transcripts were weak, while the expressions of *POU5F1* and *SOX2* transcripts were not detected. In addition, this study analyzed the expression of pluripotency-related genes and germ cell-specific gene in adherent testicular cells (Fig. 4.13A) and cultured cells for up to 1.5 month (Fig. 4.13B). The expressions of these genes were detected in adherent cells after the positive selection on the DBA and different ECM plate (Fig. 4.13A). However, the expression of POU5F1 and REX on the DBA plates was up-regulated with compared to other ECM plates at 1.5 month cultures (Fig. 4.13B).

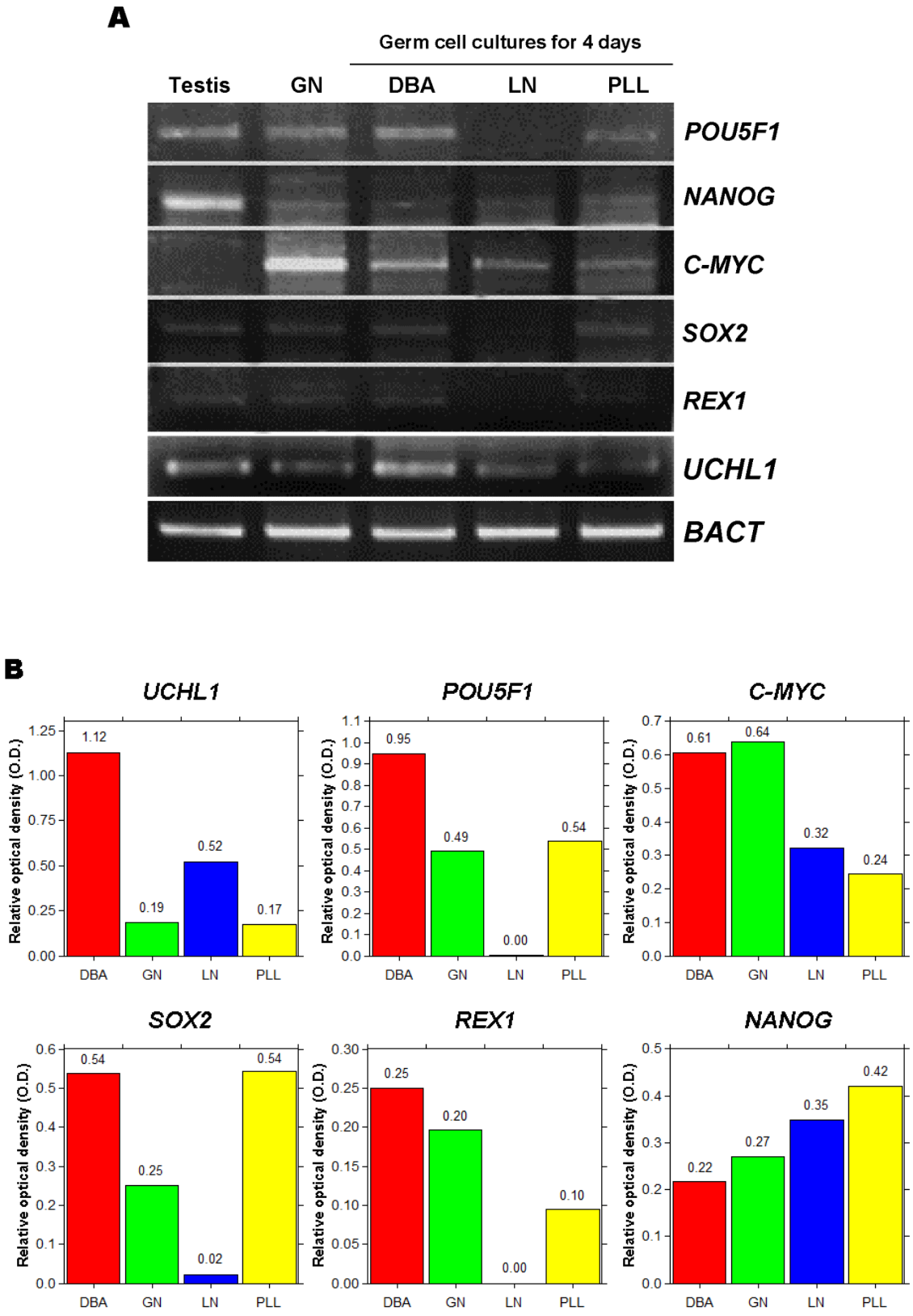

Figure 4.12 RT-PCR analysis of pluripotency-related genes in germ cells cultured on ECM and DBA plates. (*a*) PCR products on an agarose gel stained with ethidium bromide-staining. (*b-g*) Estimates of

numbers of transcripts of a germ cell-specific gene UCHL1 (*b*) and pluripotency-related genes *POU5F1* (*c*), *C-MYC* (*d*), SOX2 (*e*), REX1 (*f*) and NANOG (*g*). The DBA plate had significantly more *UCHL1* and *POU5F1* transcripts than the ECM matrix plates. In addition, *C-MYC*, *SOX2* and *REX1* transcripts were high among the ECM plates. The *C-MYC* gene was strongly expressed on the GN and DBA plates, but was not detected in 3-month old testis. Transcript levels were normalized to the abundance of *β-ACTIN* (BACT) transcripts. GN: gelatin; LN: laminin; PLL: poly-l-lysine.

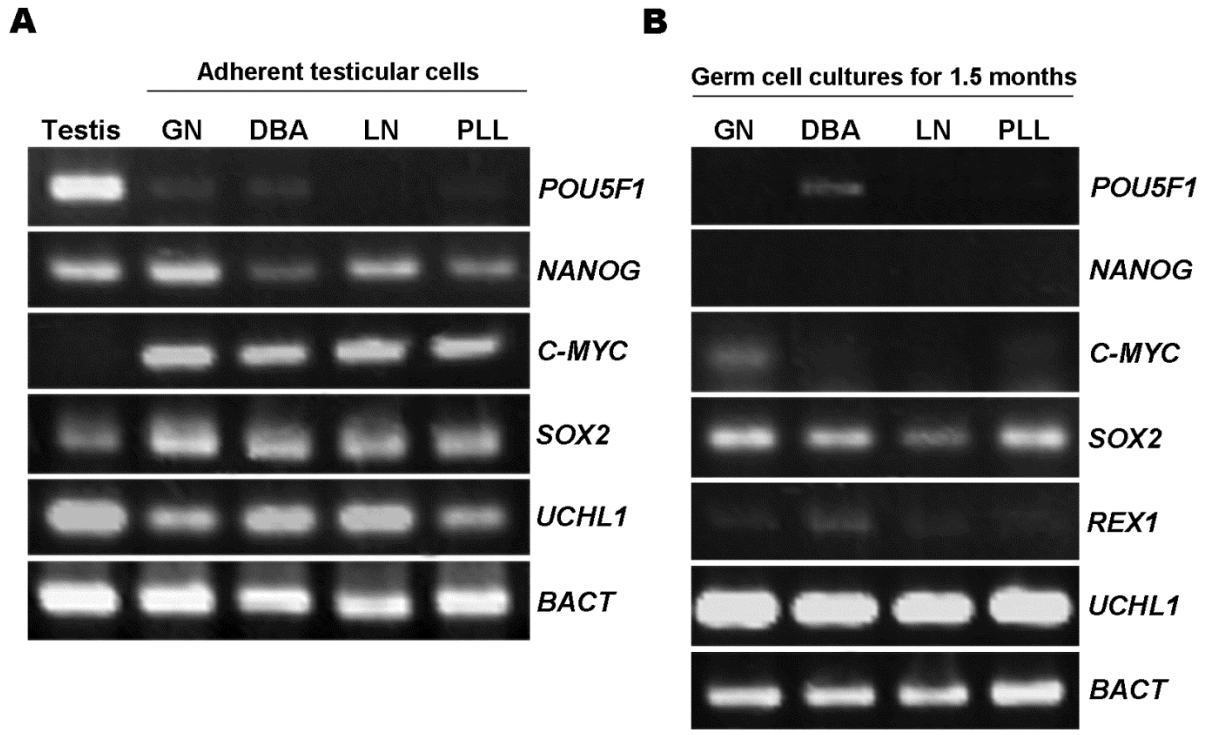

Figure 4.13 Expression patterns of pluripotency-related genes and a germ cell-specific gene in freshly collected testicular cells and cultured gem cells. (A) Freshly collected testicular cells that were incubated for 4 h on both the ECM and the DBA plates were subjected for RT-PCR analysis. The gene expressions were shown that the pluripotency-related genes (*POU5F1, NANOG, C-MYC and SOX2*) and a germ cell-specific gene *UCHL1* were expressed. (B) The transcript levels of pluripotency-related genes (*POU5F1, NANOG, C-MYC and SOX2*) and a germ cell-specific gene *UCHL1* were estimated in germ cell cultures for 1.5 months (at least 4 passages). *β-ACTIN* (BACT) was used for the loading control. GN: gelatin; LN: laminin; PLL: poly-l-lysine.

4.4 Discussion

One of the unique biological features of differentiating germ cells is their adhesion to the basement membrane of the seminiferous tubule for the abilities as a stem cell potential and the differentiation. This study investigated the effects of ECMs and DBA on the adhesion and growth of gonocytes and on their stem cell characteristics in culture.

Germ cells usually require feeder cells for their survival, proliferation and maintenance in cultures (Nagano et al., 2003). However, it was later revealed that feeder cells are not essential because they can be replaced with ECM molecules such as laminin (Kanatsu-Shinohara et al., 2005). The present study indicated that ECM molecules were not effective at enriching or purifying gonocytes from the prepubertal testis (Fig. 4.5). In addition, ES-like colony formation from gonocytes was not stimulated by the ECM molecules, but was stimulated by the presence of DBA after DBA-positive-cell selection (Fig. 4.8). ECM molecules have been used as a component of the culture medium for various types of cells. The requirement of ECM on cell survival and growth varies depending on cell types; for instance, laminin is suitable for the culture of post-migratory primordial germ cells (PGCs) (Garcia-Castro et al., 1997), gelatin is suitable for muscle cells and endothelial cells (Folkman et al., 1979; Richler and Yaffe, 1970) and poly-L-lysine is suitable for neuronal cells (Yavin and Yavin, 1980). In the present experiment, testicular cell cultures after positive cell selection resulted in different cell populations on each ECM plate (Fig. 4.9). For example, cells grown on the DBA plates mainly consisted of gonocytes with ES-cell like morphology, and cells grown on the LN plates mainly consisted of VIMENTIN-positive and epithelial-type cells, indicating that they are Sertoli cells (Herrid et al., 2007). Therefore, the cell type-specific growth pattern of testicular cells including gonocytes may be affected by ECM molecules or DBA, which are closely associated with the cell surface molecules, suggesting

that ligands for the cell surface molecules are essential components for cell adhesion and regulate physiological features of gonocytes in culture.

DBA, which recognizes α- and β-linked GalNAc residues (Kamada et al., 1991; Klisch et al., 2008), has been used to detect gonocytes and SSCs in domestic species such as pig (Goel et al., 2007) and cattle (Ertl and Wrobel, 1992; Herrid et al., 2007; Izadyar et al., 2002). DBA has also been used to enrich germ cells by magnetic-activated cell sorting (MACS) (Herrid et al., 2009), indicating that it can be a ligand for the surface glycan epitopes of germ cells. The specific affinity of the terminal GalNAc residues for their ligands may be associated with the cell surface interaction of gonocytes. Similarly, a terminal carbohydrate, such as mannose (Huang and Stanley, 2010) and *N-acetylglucosamine* (GlcNAc) (Akama et al., 2002), may be involved in the interaction between germ cells and Sertoli cells, indicating that the binding of germ cells to Sertoli cells depends on the terminal carbohydrate. Although these reports suggest that terminal carbohydrates on the surface of germ cells are associated with the cell adhesion, there is no evidence that terminal GalNAc residues are involved in the adhesion activity in the testis. At the beginning of this study, we hypothesized that GalNAc residues on the surface of gonocytes in the bovine testis that are specifically recognized by DBA affect cell survival and expansion *in vitro*. Our finding that the number of adhered gonocytes was significantly higher on the DBA-coated plate than on the ECM-coated plates (Fig. 4.5B), indicates that DBA can support the cell adhesion associated with cell survival and cell growth in cultured gonocytes.

The results shown in Fig. 4.8 indicate that the DBA-coated plates support the binding of gonocytes to the plates and result in the increased number of colonies. GalNAc residues on the surface of gonocytes are a part of Sda-glycotopes on glycoproteins, which are associated with cell surface interactions (Klisch et al., 2011). The surface interaction of terminal glycan

epitopes such as *N-acetylglucosamine* (GlcNAc)-terminated N-linked glycans, which are combined with proteins or lipids, was found to affect the adhesion and differentiation of gonocytes on Sertoli cells in mouse (Akama et al., 2002). Similarly, O-linked glycoproteins on mouse ES cells, which also have GalNAc residues and are recognized by DBA, are associated with the transition of the cells to a pluripotent state (Nash et al., 2007). The finding that masking of the terminal GalNAc residues of gonocytes by DBA pretreatment suppressed colony formation on both GN and DBA plates (Fig. 4.8A and B) indicates that the proliferation and adhesion of gonocytes can be stimulated by terminal GalNAc residues. Since structural changes of glycoproteins on a cell surface can affect cell-cell interactions and signal transduction (Dennis et al., 2009; Varki and Lowe, 2009), the formation of a GalNAc-DBA complex on gonocytes may affect cell growth, cell survival and colony formation in culture.

The ability to maintain germ cells in culture depends on the presence of supporting cells that are associated with reconstruction of the niche microenvironment (Wu et al., 2011). Sertoli cells are key somatic cells that secrete growth factors, such as glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF), which are critical factors for the self-renewal and colony formation of germ cells in mice (Kubota et al., 2004; Meng et al., 2000). The presence of Sertoli cells in cultures is known to improve the growth of germ cells (Koruji et al., 2009; Mohamadi et al., 2011). However, the flat cells surrounding the colonies of gonocytes in this experiment were mainly Sertoli cells on the LN plate that did not support colony formation (Fig. 4.8 and 4.9), while the DBA plates that suppressed the growth of somatic cells supported colony formation (Fig. 4.8). The absence of colonies on the LN plates was considered to be due to the extensive growth of testicular somatic cells that inhibited the proliferation of germ cells (Kanatsu-Shinohara et al., 2005). The higher number

of colonies on the DBA plates than on the ECM plates (Fig. 4.8B) suggests that a proper stimulation of somatic cells including Leydig cells, Sertoli cells and endothelial cells, which are necessary for survival and proliferation of germ cells (Aponte et al., 2008) supports colony formation of gonocytes on the DBA plates. In fact, the colony formation of bovine gonocytes in the culture condition can be supported on the gelatin plate at first week (Fujihara et al., 2011). However, adhesion affinity and colony formation of gonocytes was significantly lower on the GN plates than those on the DBA plates (Fig. 4.5). On the DBA plates, the colony formation abilities were maintained up to 1.5 month by increasing the number of passages (Fig. 4.11). In addition, the growth of testicular somatic cells on the DBA plates was suppressed, but not on the gelatin plates (Fig. 4.8), suggesting that DBA-coated plates may provide a suitable condition for efficient colony formation compared to gelatin plates. On the other hand, the interaction between gonocytes and Sertoli cells could not observe during the initial phase of culture (Fig. 4.6) However, Sertoli cells were associated with germ cell colonies after 7 days of culture (Fig. 4.9 and 4.10), indicating the characteristic change of gonocytes during the culture.

Colonies of bovine gonocytes have stem cell potential, as identified by the expression of stem cell-specific genes (*NANOG* and *POU5F1*) (Fujihara et al., 2011). The colony formation of testicular cells in culture depends on the presence of germ cell populations (Aponte et al., 2008), and these cell populations were strongly associated with the expression of NANOG and POU5F1 (Fig. 4.10). Transcripts of other pluripotency-related genes such as *SOX2* and *REX1* were expressed in 3 month-old bovine testis (Fig. 4.12A). The expression patterns of these genes depended on the culture plates, indicating that adhesion molecules on the plates were associated with the characteristics, including stem cell potential of germ cells in culture. The expression of most of the pluripotency-related genes (*POU5F1*, *SOX2*, *REX1*

and C-*MYC*, but not *NANOG*) was considerably increased on the DBA plates (Fig. 4.12). The expression of these genes may be required for the survival and proliferation of gonocytes. In pig, up-regulation of pluripotency-related gene in gonocytes in primary culture was shown to stimulate the proliferation and stem cell potential of the gonocytes (Goel et al., 2009) suggesting that DBA-coated plates can support the proliferation and survival of cultured bovine germ cells. Meanwhile, the expression of *NANOG* on the DBA plates was decreased, while increasing UCHL1 expression. In the bovine testis of 3-month-old, the distribution of NANOG expression showed a heterogeneity and was no longer observed in gonocytes, when they reach on the basement membrane of seminiferous tubules (Fig. 4.3, G-I), indicating that gonocytes may be intermediary stage to committee SSCs/spermatogonia. Similarly, decreasing the *NANOG* expression is observed after gonocyte phase (Mitchell et al., 2008), while the detecting UCHL1 expression in SSCs during asymmetrical division of the cells (Luo et al., 2009). Therefore, our results suggest the possibility that DBA-plates might lead to transit characteristics of gonocytes toward SSCs/spermatogonia. This hypothesis could be supported by the evidence that characteristic changes of gonocytes have been observed when they are reached at basement membrane of seminiferous tubules (Culty 2009), and the proliferative activity of cultured gonocytes seems to be induced by the transition toward SSCs/spermatogonia in mice (Kanatsu-Shinohara et al. 2005). The finding that the characteristic changes in the colony formation and the expression of pluripotency-related genes support both the growth of colonies and the differentiation of some gonocytes indicated that glycan epitope of gonocytes may associate with the further progression for the differentiation toward SSCs/spermatogonia during the postnatal developmental stage. Although a ligand of GalNAc residues was not determined in this study, the cell surface glycan epitope seems to contribute at supporting proliferation and survival on the progressive

differentiation of gonocytes in cultures. This possibility may become apparent by the analysis of proteoglycan carrying GalNAc residues and their natural receptors on Sertoli cells and/or testicular cells.

CHAPTER 5

Summary & Conclusions

This study aimed to extend the knowledge of the distribution and a functional role of GalNAc residues during the early neonatal periods of germ cell development in mammalian testes. The differential distribution of *α-N-acetylgalactosamine* (GalNAc) residues was examined on the initial differentiation stages of gonocytes by *in vivo* experiment. *In vitro* study, the role of GalNAc residues on the surface of gonocytes were examined by using their artificial ligand a lectin *Dolichos biflorus agglutinin* (DBA) in bovine germ cells.

Chapter 2 described that the presentation of GalNAc residues on the surface of gonocytes were identified by lectin-histochemistry in neonatal mouse testis. Firstly, the affinity of DBA binding to gonocytes depends on the tissue processing and the procedure for the lectin-histochemical staining. The heat-mediated antigen retrieval (HMAR) technique effectively improved DBA-binding affinity to glycan epitopes, suggesting that the dynamical modification of the glycosylation on the initial development of gonocytes can be identified by using HMAR technique, which may provide useful information during development of gem cells.

Immunohistochemical approach in Chapter 3 reveled that DBA specifically recognized GalNAc residues on gonocytes at 0.5 to 1 dpp, but was gradually disappeared at around 3-5 dpp. The loss of DBA affinity to gonocytes may be due to the change of a terminal glycosylation on the cell surface, considering that the alteration of glycosylation may reflect the physiological transition of gonocyte to committee onset of spermatogenesis. Double-immunolocalization showed DBA signals were intensely observed as condensed dots in the

cytoplasm of gonocytes. Interestingly, these germ cells were progressively lost the expression of germ cell-specific marker UCHL1 at around 5 to 7 dpp, and then they expressed a premeiotic marker STRA8 at 7 dpp. These observations suggesting that the change of glycosylation on gonocytes was associated with the appearance of differentiating spermatogonia, which are eventually differentiated into the premeiotic stage of spermatogenesis. Meanwhile, DBA-negative cells still expressed UCHL1, but did not express STRA8 at the same stage. These molecular features indicate that gonocytes consist of a heterogeneous population and the modification of glycan epitopes requires for the progressive differentiation of spermatogenesis.

In vitro study in Chapter 4 examined that whether the interaction of glycan epitopes on the surface of bovine gonocytes with DBA or extracellular matrix (ECM) components such as gelatin (GN), laminin (LN) and poly-L-Lysine (PLL) associated with their abilities as a stem cells potential and differentiation *in vitro* condition. The results demonstrated that the adhesion efficiency of bovine gonocytes on DBA plates was significantly higher with compared to ECM plates. Gonocytes successfully formed mouse embryonic stem (ES)-like cell colonies in both DBA and GN plates, and the average numbers of colonies in cultures significantly increased on DBA plates than other ECM components. Interestingly, pretreating gonocytes with DBA to neutralize the terminal GalNAc residues strongly suppressed colony formation. In contrast, the DBA plate was effectively restricted the growing of somatic cells, resulting in a beneficial effect for colony formation. These results suggest that DBA can support the cell adhesion associated with cell survival and cell growth in cultured gonocytes.

The levels of transcripts in gonocyte cultures showed that the expression of pluripotency-related transcription factors were dynamically changed on the lectin DBA and different ECM plates, including stem cell potential of germ cells in culture. The expression of

most of the pluripotency-related genes (*POU5F1*, *SOX2*, *REX1* and C-*MYC*, but not *NANOG*) was considerably increased on the DBA plates. The finding that the expression of germ-cell marker *UCHL1* was markedly increased on the DBA plates indicates that the gonocytes were enriched on the DBA plates. These results suggest that these a structural change of carbohydrate chains by forming GalNAc-DBA complex provide a suitable microenvironment for cell adhesion and colony formation of spermatic stem cells and provide a possibility to maintain bovine gonocytes for long-term culture.

This study suggested that glycan epitopes on the surface of gonocytes closely associated with the determination of the cell fate during the initial differentiation periods committed to undifferentiating spermatogonia and/or differentiated spermatogonia (Fig. 5.1). These glycan epitopes play a role as adhesion molecules, which may regulate the cell survival and behaviors through the carbohydrates-mediated signal transduction corresponding to glycoproteins and glycolipids (Fig. 5.1).

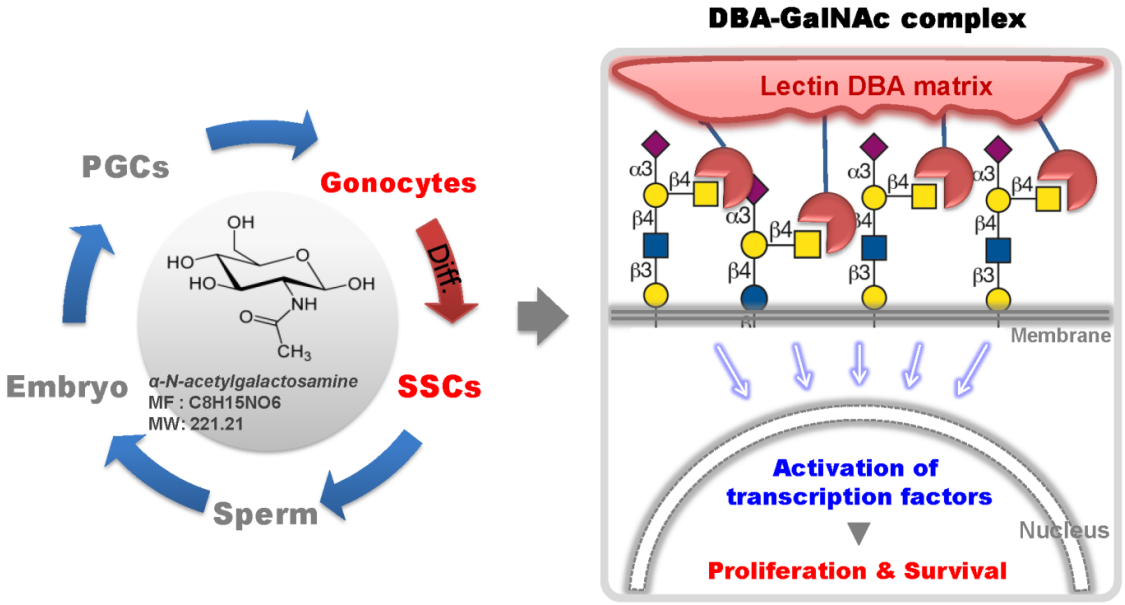

Figure 5.1 Schematic representation of the role of GalNAc-mediated signal transduction on the surface of gonocytes in mammalian species.

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